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Full Length Research Paper

Effects of organic nitrogen and carbon sources on mycelial growth and polysaccharides production and their optimization in the submerged culture of *Grifola umbellate*, a Chinese medicinal herb

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Grifola umbellate **is a famous and expensive Chinese herb medicine and the main medicinal component is polysaccharide mainly produced by its mycelia. Effects of organic nitrogen and carbon resources on mycelial growth and polysaccharides production of a medicinal mushroom, *G. umbellate* were studied in the submerged culture system. Soybean meal was selected as the optimal organic nitrogen source for its significant promotion effects on mycelial growth and polysaccharides synthesis based on results of an orthogonal matrix experiment design. Glucose was proved to be a preferred carbon source by comparison with fructose and sucrose. The optimal dosages of soybean meal 5.63% (w/v) and glucose 4.33% (w/v) were obtained, respectively through the curvilinear regressions experiment. The productions of mycelial dry weight, exo-polysaccharide (EPS) and inner polysaccharide (IPS), in the optimal medium, were about 3.16, 1.48 and 2.78 folds higher than those in the basal medium, respectively and culture duration was shortened to half through optimizing the organic nitrogen and carbon sources. These results will be significant contribution to process biochemistry of medicinal fungus in the submerged culture system.**

Key words: *Grifola umbellate,* Chinese medicinal herb. polysaccharides, submerged culture, organic nitrogen source, carbon source, orthogonal design.

INTRODUCTION

Grifola umbellate (Pers. ex Fr.) Pilat, “zhuling” in Chinese, is a *Basidiomycete* fungus belonging to the family Polyporaceae. Its fruiting body growing above-ground is a delicious edible mushroom and its sclerotium growing under-ground has been popularized as a traditional Chinese medicine using for treatments of inhibited urination, beriberi, edema and other diseases for more than 2000 years in Chinese history.

The bioactive components of *G. umbellate* sclerotium are various water-soluble polysaccharides. These polysaccharides are β-D-glucans, which are built up with β-(1→3), β-(1→4) and β-(1→6) links. Their average molecular weight ranges within 1~70000 and intrinsic viscosity is 14.9 ~ 22.9 (Miao and Li, 2000). Based on the development of modern medicine and pharmacological research of polysaccharides, the medical value of *G. umbellate* polysaccharide has been recognized and used for treatment of blood pressure regulation (Ji, 2005), immuno-modulation (Xu et al., 2003) and suppression of

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Table 1. $L_9(4^3)$ orthogonal design of optimization nitrogen source components.

Factors (w/v)	Soybean meal (SM)	Peptone (PT)	Yeast extract (YE)	Blank
Level 1	0	0	0	1
Level 2	1%	0.1%	0.1%	2
Level 3	3%	0.5%	0.5%	3

tumor growth (Chen and Li, 2004). In recent years, many clinical studies showed the therapeutic alliance of *G. umbellate* polysaccharides and chemical medicines could enhance the therapeutic efficacy and reduce the side effects of the chemical counterparts (Lv et al., 2005; Zhang et al., 2007).

Either growing naturally or cultured artificially, the maturation period of *G. umbellate* sclerotia is long (usually three to four years). Furthermore, both quality and yield of sclerotia were instable due to the influence of environmental factors. So, there is an urgent demand for a feasible alternative to obtain the bio-active polysaccharides. Since the solid-state culture couldn't provide high yields of bio-active polysaccharides, the submerged culture becomes the only choice to produce mycelia and polysaccharides on a larger scale.

Recent studies have shown that the main ingredients of the polysaccharides extracted from *G. umbellate* sclerotia and submerged culture mycelia are the same (Xu et al., 2004). Experimentations on animal subjects have also proven that both of them displayed the inhibition for sarcoma cells, lung and liver cancers of mice and exhibited strong immuno-modulatory effects (Dai et al., 1979; Zhou et al., 2001; Li et al., 2007). At present, polysaccharides of *G. umbellate* have been applied in the clinical therapies of chronic viral hepatitis, psoriasis, hepatitis B and other diseases with good therapeutic effects (Wang et al., 2000).

Currently, available reports on *G. umbellate* submerged cultures have disagreements on the composition of the optimal medium (Zhou et al., 2001; Gu et al., 2001; Chen et al., 2002). Most studies results supported that adding corn meal as basal medium composition can promote *Basidiomycete* fungus growth, but the views on carbon and nitrogen sources use were divergent markedly. Moreover, an obvious response relation between the intensity of *G. umbellate* mycelia growth and the content of organic nitrogen in the culture medium was found in our study. To the best of our knowledge, no report has mentioned that *G. umbellate* can survive in any rich nutrient medium, comparing with normal medium. So, further study on the compositions of the submerged culture medium is necessary.

The propose of this study is to determine the optimal nutritional requirements for *G. umbellate* mycelial growth and polysaccharides productions in the submerged culture and describe the correlation between productions of mycelia and polysaccharides and the dosages of nitrogen source and carbon source in the medium by a curvilinear regression method.

MATERIALS AND METHODS

Microorganism and inoculum

The CB-2 strain of *G. umbellate* used in this study was obtained from Northwest A&F University, China. The strain was cultured on Potato Dextrose Agar (PDA) slants at 25℃ for 14 days, then stored at 4℃ and sub-cultured every 3 months.

The mycelium was activated by culturing at 25℃ for 5 days and then inoculated on a modified agar plate which consisted of the following component: 30 g/L core meal, 20 g/L glucose, 1.0 g/L yeast extract, 1.0 g/L peptone, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ and 15 g/L agar. The agar plates were incubated at 25℃ for 10 days. Four pieces of activated mycelial discs about 6 mm in diameter were punched out with a sterilized cutter and transferred to the seed submerged culture medium (plate medium without agar). Then the inoculums were grown in a 250 ml flask containing 100 ml medium and incubated at 25℃ on a rotary shaker at 150 rpm for 10 days.

Flask culture conditions

The flask culture experiments were performed in 250 ml flasks containing 100 ml of fermentation medium after inoculating with 10% (v/v) of the seed culture medium. The basal submerged medium was similar to the seed submerged culture medium except that the initial pH was adjusted to 5.5 using 1 N NaOH and 1 N HCl. The culture was incubated at 25℃ on a rotary shaker incubator at 150 rpm and 100 ml samples were collected for mycelia dry weight, exo-polysaccharide (EPS) and inner polysaccharide (IPS) productions analysis.

Effects of the varieties and concentrations of organic nitrogen sources on *G. umbellate* culture were studied by varying the concentrations of soybean meal (SM), peptone (PT) and yeast extract (YE) in the medium. Soybean extract was obtained from the meal using distilled water at 60℃ for 1 h and was used as a nitrogen source. Media were sterilized at 121℃ for 30 min while glucose was autoclaved separately. An orthogonal $L_9(4^3)$ was used to obtain the optimal nitrogen source components in the submerged culture for soybean meal, peptone and yeast extract. The levels of components of $L_9(4^3)$ orthogonal design are listed in Table 1.

To find the suitable carbon sources, glucose, fructose and sucrose were separately employed in the basal medium and their influences on *G. umbellate* mycelial dry weight and polysaccharides productions were studied. The optimal dosages of the most suitable organic nitrogen and carbon sources were determined by the curvilinear regression experiments.

Analytical methods

Mycelia concentration was represented by the mycelia dry weight (MDW) per unit volume. 100 ml of fermentation broth was centri-

Table 2. Results of $L_9(4^3)$ orthogonal test of mycelial biomass and polysaccharides productions of *G. umbellate* in shake flask culture.

Run	SM %	PT %	YE %	Blank	MDW (g/L)	EPS (g/L)	IPS (g/L)
1	0	0	0	1	2.49 ± 0.12	1.68 ± 0.15	0.12 ± 0.01
2	0	0.1	0.1	2	12.00 ± 0.31	0.82 ± 0.02	0.70 ± 0.01
3	0	0.5	0.5	3	12.81 ± 0.44	1.28 ± 0.17	0.84 ± 0.06
4	1	0	0.1	3	13.10 ± 0.25	0.67 ± 0.01	0.26 ± 0.02
5	1	0.1	0.5	1	16.59 ± 0.53	1.02 ± 0.03	1.14 ± 0.02
6	1	0.5	0	2	15.93 ± 0.41	0.94 ± 0.11	0.84 ± 0.03
7	3	0	0.5	2	17.42 ± 0.58	3.40 ± 0.03	2.32 ± 0.10
8	3	0.1	0	3	18.16 ± 0.72	1.52 ± 0.02	2.03 ± 0.12
9	3	0.5	0.1	1	21.75 ± 0.36	1.78 ± 0.02	1.83 ± 0.05

SM, PT and YE represent factors of soybean meal, peptone and yeast extract, respectively. Values are means ± SD of triple determinations.

fuged at 4000 rpm for 30 min. Then washed mycelia pellets repeatedly with distilled water and dried at 60°C until a constant weight was achieved. The supernatant was mixed with a four volumes of 95% ethanol, stirred vigorously and left overnight at 4°C. Precipitated exo-polysaccharide (EPS) was centrifuged at 10,000 g for 20 min and the supernatant was discarded. The precipitate was then washed with 100% ethanol and ethyl ether. After drying, the content of crude EPS was estimated by a phenol-sulfuric acid method using glucose as the standard (Dubois et al., 1956). The residual sugar concentration was measured by a 3, 5-dinitryl-salicylic acid colorimetry method (Cai and Yuan, 1982)

The inner polysaccharide (IPS) was extracted from dried mycelia powder (200 mg) by suspending the mycelia in 5 ml distilled water at 90 ± 1°C for 1 h with gentle stirring (Hromádková et al., 1999) and the procedure was repeated for 3 times. The extracted slurry was filtered and the content of IPS was determined by the procedure used for EPS measurement as described above. In this paper, EPS production (g/L) was represented with the exo-polysaccharide content in a liter of culture solution and IPS production (g/L) was represented with the inner polysaccharide content of mycelia in a liter of culture solution. The pH value of culture filtrate was determined using a digital pH meter.

Statistical analysis

All the experiments were performed in triplicate and the software package SPSS 9.0 was used to analyze the data.

RESULTS

Optimization of the organic nitrogen source composition

Shih suggested that most basiomycetes prefer organic nitrogen sources for submerged culture to inorganic nitrogen sources, because they can synthesize certain essential amino acids in the cultivation of higher fungi (Shih et al., 2006). Then soybean meal, peptone and yeast extract were investigated using an orthogonal layout $L_9(4^3)$ as listed in Table 1. The concentrations of organic nitrogen sources in this experiment were based on the results of previous studies (Zhou et al., 2001; Chen et al., 2002). Those organic nitrogen sources were employed into basal medium based on Table 2 and the experiment results were listed in the last three columns in Table 2.

According to the analyzed results of this orthogonal test, the order of effects of organic nitrogen source on mycelial growth and EPS production were SM > PT > YE, while the order of effects on IPS production was SM > YE > PT.

Variance analysis was used to test the effects of nitrogen source and the results were shown in Table 3. Only soybean meal had significant effect on the mycelial growth and IPS production. It implied that peptone and yeast extract would be unnecessary for *G. umbellate* submerged culture. Relatively high levels of mycelial dry weight and polysaccharides productions were achieved when soybean meal was used at 3% (w/v). However, it did not confirm that 3% (w/v) should be the most suitable dosage. Therefore, the optimal dosage of soybean meal was explored in the following experiments.

Range of soybean meal dosage

When 3, 10, 30 and 50 (w/v) soybean meal were employed in the basal medium as the organic nitrogen sources, respectively, we found that *G. umbellate* cannot survival in the medium which 50% (w/v) soybean meal was used. However, the maximum level of mycelial dry weight (32.97 g/L) and IPS production (5.94 g/L) were obtained at the same time, when soybean meal was used at 10% (w/v); while the maximal EPS production (5.32 g/L) was gained at 30% (w/v). Therefore, the optimal dosage of soybean meal in submerged culture medium should be in the range of 3~30% (w/v). Effects of the soybean meal dosage on the mycelial growth and polysaccharides productions were studied in another experiment.

The smooth curves fitting mycelial dry weight, EPS

Table 3. Results and variance analysis of L_9 (4^3) orthogonal experiment on optimal organic nitrogen sources of *G. umbellate* in shake flask culture.

Parameter	MDW (g/L)				EPS (g/L)				IPS (g/L)			
	SM	PT	YE	Blank	SM	PT	YE	Blank	SM	PT	YE	Blank
k_1	9.10	11.00	12.19	13.61	1.26	1.92	1.38	1.49	0.55	0.90	1.00	1.03
k_2	15.21	15.58	15.62	15.12	0.88	1.12	1.09	1.72	0.75	1.29	0.93	1.29
k_3	19.11	16.83	15.61	14.69	2.23	1.33	1.90	1.16	2.06	1.17	1.43	1.04
R	10.01	5.83	3.42	1.50	1.36	0.80	0.81	0.56	1.51	0.39	0.51	0.26
Degree of freedom	2	2	2	2	2	2	2	2	2	2	2	2
Sum of squares	152.78	56.47	23.36	3.61	2.94	1.03	1.01	0.48	4.01	0.24	0.45	0.13
Mean square	76.39	28.24	11.68	1.81	1.47	0.51	0.50	0.24	2.01	0.12	0.23	0.06
F-ratio	42.32*	15.64	6.47	1.00	6.13	2.14	2.10	1.00	30.88**	1.85	3.49	0.96

$K^X_i = K^X_i/3$ ($K^X_i = \sum$ mycelial biomass or polysaccharides in three replications at X_i); R^X_i = max k^X_i -min k^X_i; Values are mean ± SD of triple determinations; Symbol *X* represents SM, PT and YE, respectively, and symbol *i* represents each level.
Symbol * represents $F_{ratio} > F_{0.1}$; symbol ** represents $F_{ratio} > F_{0.01}$.

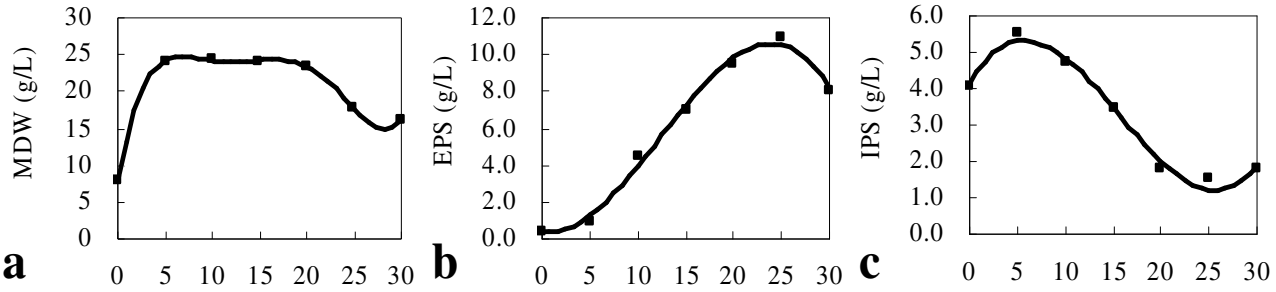

Figure 1. The relationship of soybean meal dosage and the productions of mycelia and polysaccharides in *G. umbellate* submerge cultivation: (a) Mycelial dry weight, (b) EPS production, (c) IPS production.

production or IPS production with regards to the soybean meal dosage was shown in Figure 1. The optimal dosage of soybean meal could be obtained according to curvilinear regression (Xu, 1998; Wu et al., 2006).

As shown in Figure 1, MDW had no significantly difference when soybean meal dosage is changed from 5 to 20% (w/v) in the medium. The curvilinear regressions of soybean meal dosage are shown respectively as follows: for mycelial dry weight, $y_1 = 0.00004x^5 - 0.0032x^4 + 0.0926x^3 - 1.2347x^2 + 7.5164x + 7.7501$; for EPS production, $y_2 = -0.0018x^3 + 0.0695x^2 - 0.2327x + 1.0757$ and for IPS production, $y_3 = 0.001x^3 - 0.0487x^2 + 0.4534x + 4.1321$. Computed by the curvilinear regression equations, the maximum MDW (24.63 g/L) could be achieved when 6.68% (w/v) soybean meal dosage is used and the maximal EPS production (10.64 g/L) and maximal IPS production (5.32 g/L) also could be gained when 23.94% (w/v) and 5.63% (w/v) soybean meal is used in submerge medium, respectively. In this study, 5.63% (w/v) was selected as the optimal dosage of soybean meal to obtain the maximal production of IPS. And according to the curvilinear regression equations, it was predicted that MDW 24.46 g/L, EPS 1.64 g/L and IPS 5.32 g/L could be obtained by employing 5.63% (w/v) soybean meal in *G. umbellate* medium.

Effect of different carbon sources

According to the results of previous studies (Zhou et al., 2001; Li et al., 2007; Wang et al., 2000; Gu et al., 2001), glucose, fructose and sucrose were proved to be more suitable than other carbon sources. Therefore, those three kind sugars were separately employed in the medium at 3% (w/v), in which 5.63% (w/v) soybean meal was added in advance. Among the sources examined, glucose yielded the maximum mycelial dry weight (17.57 g/L) and the maximum IPS production (3.83 g/L), where as sucrose yielded the maximum EPS production (8.43 g/L). It indicated that glucose was beneficial to the mycelia growth and IPS synthesis, but sucrose was benefit to the for EPS synthesis. Such a phenomenon was also found in submerged cultivation of other kinds of mushrooms (Hwang et al., 2003; Wei et al., 2008). From the practical point of view, glucose is an optimal carbon source for *G. umbellate* submerged culture because of its economy and high yield in industrial production. Therefore, glucose was selected as the carbon source in the

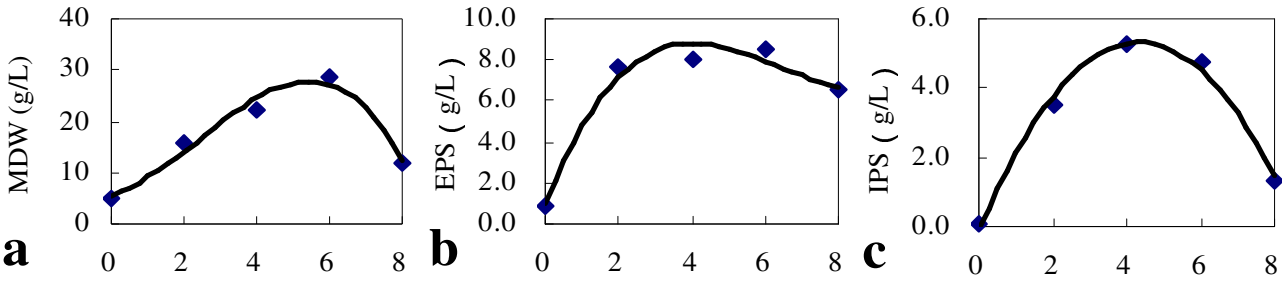

Figure 2. The relationship of glucose dosage and the productions of mycelia and polysaccharides in *G. umbellate* submerged cultivation: (a) Mycelial dry weight, (b) EPS production, (c) IPS production.

following experiments in order to maximize the production of IPS.

Range of glucose dosage

In order to study the optimal dosage of glucose, 5.63% (w/v) soybean meal and different concentrations of glucose (ranging from 0 to 8% (w/v)) were added to separate flasks. The effects of glucose on the mycelial growth and polysaccharides productions were studied using the same method described in section 3.2 and the results were shown in Figure 2.

The curvilinear regressions of glucose dosage are shown respectively as follows: for mycelial dry weight, $y_1 = -0.2009x^3 + 1.4363x^2 + 2.2318x + 5.549$; for EPS production, $y_2 = 0.0433x^3 - 0.832x^2 + 4.6016x + 0.9467$ and for IPS production, $y_3 = -0.2877x^2 + 2.4917x - 0.076$. Computed by the curvilinear regression equation, the maximum MDW (27.85 g/L) would be achieved when 5.45% (w/v) glucose is added into the medium and the maximum EPS production (8.82 g/L) and IPS production (5.32 g/L) would be gained when 4.04% (w/v) and 4.33% (w/v) glucose is used, respectively. Therefore, 4.33% (w/v) could be selected as the optimal dosage of glucose based on the IPS production. According to the curvilinear regression equations, it is predicted that MDW 25.83 g/L, EPS 8.79 g/L and IPS 5.32 g/L would be obtained by employing 4.33% (w/v) glucose in *G. umbellate* medium.

Comparison of basal and optimal culture medium

To investigate the kinetic mode and yield on the mycelia and polysaccharides productions, *G. umbellate* was cultivated in flask under the basal culture medium (30 g/L core meal, 20 g/L glucose, 1.0 g/L yeast extract, 1.0 g/L peptone, 1 g /L KH_2PO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$) and optimal culture medium(30 g/L core meal, 43.3 g/L glucose, 56.3 g/L soybean meal, 1 g/L KH_2PO_4 and 0.5 g/L $MgSO_4 \cdot 7H_2O$), under the same condition. As shown in Figure 3a, in the basal culture medium, the production of MDW reached maximum yield (9.66 g/L) at 21 d of the cultivation while the productions of EPS and IPS reached 7.89 and 2.37 g/L at 21 d and 24 d, respectively. However, the production of MDW reached maximum (30.50 g/L) at 9 d in the optimal culture medium (Figure 3b), while the productions of EPS and IPS reached 11.72 and 6.60 g/L at 11 d and 13 d, respectively. It showed that the optimization of the organic nitrogen and the carbon sources could increase producing rate of mycelia and polysaccharides and significantly shorten the duration of the submerged culture.

DISCUSSION

G. umbellate is a specific medical fungus, because it can grow vigorously in high nutritional conditions. To date, no report was available regarding this characteristic of any species of basidiomycete. Therefore, our study solely concentrated on selecting more suitable nitrogen and carbon source so as to improve the mycelial and polysaccharides productions and determining their optimal dosages by using regression curves method in particular (Zhou et al., 2001; Chen et al., 2002; Shih et al., 2006; Xu, 1998; Wu et al., 2006).

This study indicates that soybean meal is an appropriate organic nitrogen source for improving mycelial and polysaccharides productions in *G. umbellate* submerged culture. The maximum of mycelial dry weight, EPS production and IPS production could be gained when the soybean meal was employed at 6.68, 23.94 and 5.63% (w/v), respectively. The results also showed that glucose is beneficial to the mycelia growth. For the maximal IPS production, the optimal dosage of glucose and soybean meal was 4.33 and 5.63% (w/v) respectively (Hwang et al., 2003; Wei et al., 2008).

Compared with the basal medium, the duration of *G. umbellate* submerge culture could be shortened from 24 d to 13 d by using the optimized medium and the mycelial dry weight, EPS production and IPS production could be increased about 3.16, 1.48 and 2.78 folds, respectively. Besides, our experiments proved that feeding 5.63% (w/v) soybean meal and 4.33% (w/v) glucose into *G. umbellate* submerged culture medium is suitable (Xu et

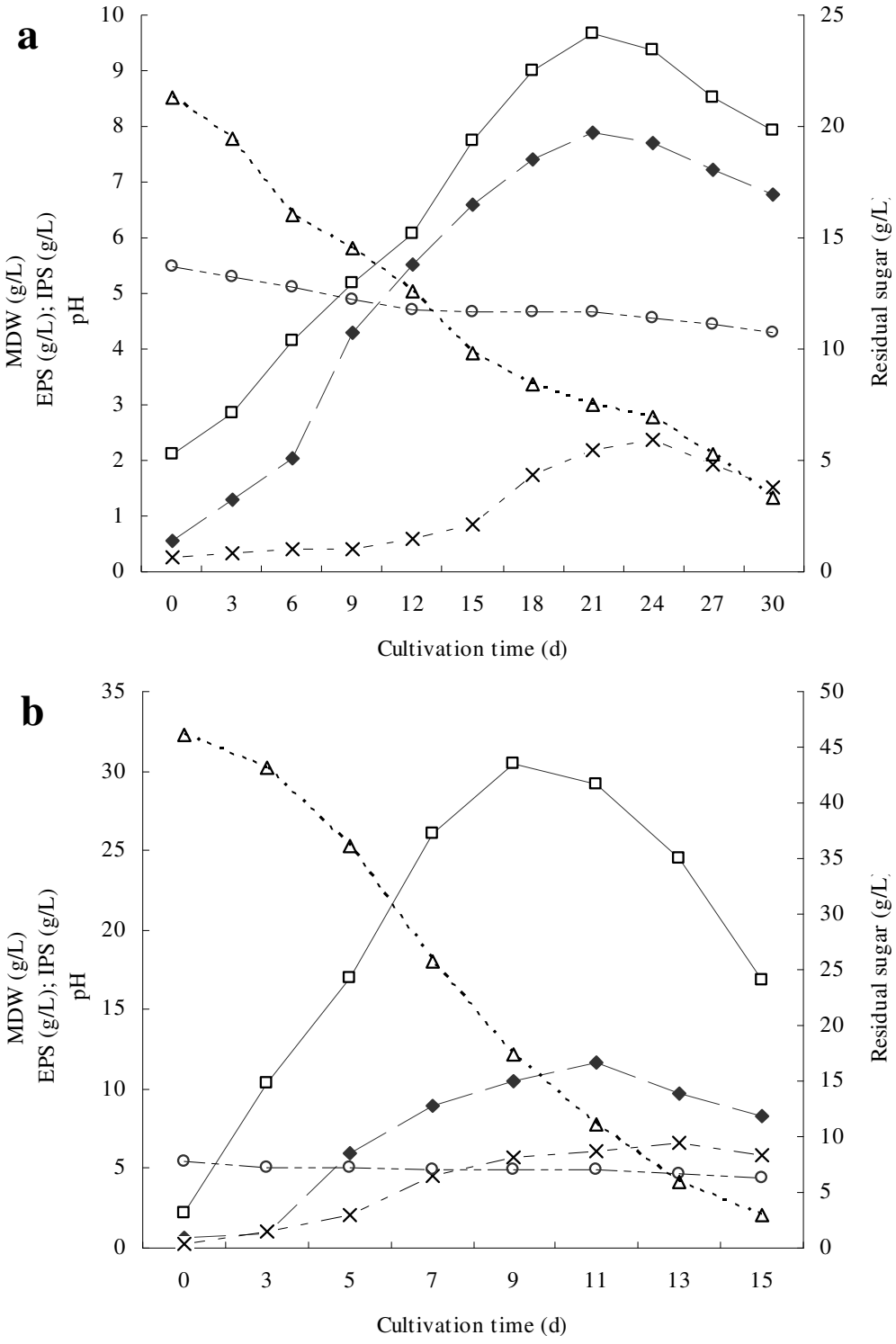

Figure 3. Time profile of mycelial growth and polysaccharides productions of *G. umbellate*: (a) under the basal culture medium, (b) under the optimal culture medium. (ϒ) Mycelial dry weight, (∅) EPS production, (×) IPS production, (≥) Residual sugar, (○) pH.

al., 2003; Chen and Li, 2004; Lv et al., 2005; Zhang et al., 2007; Hwang et al., 2003; Wei et al., 2008).

On the other hand, we found that the optimal dosage of soybean meal was different depending on different target products (e.g. MDW, EPS or IPS). We suggested that the selection of appropriate soybean meal dosage should be

based on target product in industrial production. In the same way, it should be considered to use sucrose as carbon source in order to obtain the maximum EPS production.

Furthermore, it is necessary to validate the fermentation yield in a bioreactor using optimized organic nitrogen and carbon sources to achieve the demand of large-scale production, which is an ongoing project in our laboratory.

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