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Nutritional Requirements for the Mycelial Biomass and Exopolymer Production by *Hericium erinaceus* CZ-2

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Summary

In this work, the effects of medium composition and fermentation parameters on the simultaneous production of mycelial biomass and exopolymer by medicinal mushroom *Hericium erinaceus* CZ-2 were investigated in shake flask cultures using one-factor-at-a-time method and orthogonal array design. Results showed that the most suitable carbon, nitrogen, mineral sources, and cofactors for the mycelial biomass and exopolymer production were: corn flour combined with 1 % glucose, yeast extract, KH_2PO_4 and corn steep liquor. The intuitive analysis of orthogonal array design results indicated that the effects of nutritional requirement on the mycelial growth of *Hericium erinaceus* CZ-2 were in regular sequence of corn flour combined with 1 % glucose > yeast extract > corn steep liquor > KH_2PO_4, and those on exopolymer production were in the order of corn flour combined with glucose > KH_2PO_4 > yeast extract > corn steep liquor. The maximal yield of mycelial biomass (16.07 g/L) was obtained when the composition of the culture medium was (in g/L): corn flour 30, glucose 10, yeast extract 3, KH_2PO_4 1, $CaCO_3$ 0.5, and 15 mL/L of corn steep liquor; while the maximal exopolymer yield (1.314 g/L) was achieved when the composition of medium was (in g/L): corn flour 30, glucose 10, yeast extract 5, KH_2PO_4 3, $CaCO_3$ 0.5, and 15 mL/L of corn steep liquor. In the 15-litre scale-up fermentation, the maximum mycelial biomass yield of 20.50 g/L was achieved using the optimized medium.

Key words: Hericium erinaceus, submerged culture, medium optimization, mycelial biomass, exopolymer, orthogonal array design method

Introduction

Hericium erinaceum is a basidiomycete fungus taxonomically belonging to the family Hericiaceae, order Hericiales, division Homobasidiomycetes, class Hymenomycetes (*1*). Its fruiting body is called houtou in Chinese due to its shape resembling monkey's head, and has been used as an edible and medicinal fungus in China and other oriental countries and areas for many years (*2*). *Hericium erinaceum* has recently attracted considerable attention for its various physiological activities, such as antitumor, antioxidant, hypoglycemic, hypocholesterolemic and immunostimulating activities; and its various biological compounds such as polysaccharides, extracted from the fruiting bodies and cultured mycelia, and exopolymers produced with submerged culture have been reported as well (*3–8*).

The systematic research of pharmacological effects of *Hericium* started only about 20 years ago. Its cultivation

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on solid substrates or submerged cultivation has become essential to meet the increasing demands on the markets. Traditionally, *Hericium* has been reported to be cultured on the solid substrate for several months to obtain its fruiting bodies using composts or lignocellulosis wastes such as straw or wood. Moreover, submerged culture has shown its advantage due to its shorter cultivation period, consistent quality of the product, and simultaneous outgrowth of exopolymers, such as polysaccharide, glycoprotein or other proteins, which also exhibit various biological effects (*5,8,9*).

There are many reports on optimization of medium composition or culture conditions for mushroom fermentation producing bioactive metabolites by various statistical optimization techniques such as response surface methodology or uniform design method (*10–14*). However, there have been no detailed reports on the effects of submerged culture conditions and medium composition on simultaneous production of mycelial biomass and exopolymer by fungus *Hericium erinaceus* untill now. Hence, in the present article, we mainly investigated the effects of various factors such as culture operating parameters and medium composition on the production of mycelial biomass and exopolymer from *H. erinaceus* CZ-2.

Materials and Methods

Microorganism and medium

The strain of *Hericium erinaceus* CZ-2 was isolated from the mountainous district of Anhui Province in China and was kept in the Institute of Bioengineering. The stock culture was maintained on potato dextrose agar (PDA) slants and subcultured every two months. The slants were incubated at 25 °C for 7 days. The seed culture medium was composed of (in g/L): glucose 10, yeast extract 3, KH_2PO_4 1, and $MgSO_4 \cdot 7H_2O$ 0.5.

Inoculum preparation and flask culture

For preparation of the inoculum, 10 pieces (about 5 mm) of the mycelia of *Hericium erinaceus* CZ-2 were transferred from a slant into each 250-mL Erlenmeyer flask containing 50 mL of the above mentioned seed medium.

The flask culture experiments were performed in a 500-mL flask containing 150 mL of the fermentation medium after inoculation with 10 % (by volume) of the mycelium suspension at 25 °C on a rotary shaker incubator at 150 rpm for 7 days. The fermentation medium was based on the basal medium (with the composition of (in g/L): glucose 10, yeast extract 3, KH_2PO_4 1, and $MgSO_4 \cdot 7H_2O$ 0.5, dissolved in distilled water, with the initial pH=7.0) to screen the most suitable carbon and nitrogen sources, mineral element, *etc.* Triplicate experiments were also carried out, and the mean values were given. All data were subject to variance analysis and expressed as mean ± S.D.

Batch fermentation in 15-litre fermentor

The batch fermentation was carried out in a 15-litre fermentor equipped with three 6-bladed disc impellers (Biostat C10-3, B. Braun, Germany), oxygen and pH electrodes, under the following conditions: working medium volume 10 L, inoculation volume 10 % (by volume), culture temperature 25 °C, aeration rate 0.8 vvm, and agitation speed 80 rpm. The fermentation continued until the mycelial biomass or exopolymer reached their highest values.

Analytical methods

As described previously by Cui *et al*. (*10*), the mycelia were harvested at the end of fermentation, then centrifuged at 12 000×*g* for 10 min, and dried to constant mass at 60 °C in laboratory vacuum ovens. The supernatant was filtered through a 0.45-μm membrane filter (Millipore, USA). The resulting filtrate was concentrated and mixed with absolute ethanol, 3 times of the original volume. The mixture was stirred vigorously and then maintained at 4 °C overnight. The precipitated exopolymer was lyophilized and its mass was measured. Reducing sugar was measured with the DNS method.

Experimental design

Culture conditions and medium composition experiments were designed to meet the nutritional demand of mycelial growth and exopolymer production by *H. erinaceus* CZ-2. The carbon source, nitrogen source, mineral sources, growth cofactors and initial pH (pH value of unsterilized culture broth) were regarded as correlated factors of the culture medium, in particular carbon and nitrogen sources. According to the results of one-factor--at-a-time experiments, the orthogonal array design method $L_9(3^4)$ was selected to optimize the medium composition. Tables 1 and 2 give the factors and their levels, and the experimental orthogonal design, respectively. Important factors leading to the best possible response were conducted from the calculation results in Table 2.

Table 1. $L_9(3^4)$ experimental factors and their related levels for orthogonal array design

Level	A/(g/L) Corn flour +1 % glucose	B/(g/L) Yeast extract	C/(g/L) KH_2PO_4	D/(mL/L) Corn steep liquor
1	20	1	1	5
2	30	3	3	10
3	40	5	5	15

Results and Discussion

Effects of cultural conditions

The two factors, initial pH and culture temperature, were commonly considered as the significant factors that affect mushroom fermentation process. Therefore, *Hericium erinaceus* CZ-2 was cultivated in basal medium at pH values ranging from 3 to 10 to find the optimal initial pH for mycelial growth and exopolymer production (data not shown). The optimal initial pH for mycelial growth and exopolymer production was between 5 and 6, corresponding to the mycelial yield and exopolymer production of 10.56 and 0.854 g/L, respectively, which was similar to the results obtained with other mushrooms in slightly acidic pH conditions (*15,16*).

Table 2. Orthogonal array design and the responses of the dependent variables (biomass and exopolymer) produced by *Hericium erinaceus* CZ-2 in shake flask culture

Run		Independent variables				Dependent variables	
		A	B	C	D	γ(biomass)/(g/L)	γ(exopolymer)/(g/L)
1		1	1	1	1	9.21±0.44	0.550±0.035
2		1	2	2	2	12.66±0.88	1.017±0.046
3		1	3	3	3	11.88±0.76	0.931±0.011
4		2	1	2	3	12.86±0.40	1.275±0.034
5		2	2	3	1	14.10±0.65	1.175±0.121
6		2	3	1	2	14.79±0.72	1.088±0.007
7		3	1	3	2	11.15±0.21	0.983±0.016
8		3	2	1	3	14.57±0.57	0.894±0.039
9		3	3	2	1	12.45±0.31	1.136±0.009
Mycelial biomass	K1	33.22	33.75	38.57	35.76	The order for mycelial biomass: corn flour combined with 1 % glucose > > yeast extract > corn steep liquor > KH_2PO_4	
	K2	41.34	41.75	37.97	38.60		
	K3	39.12	38.17	37.13	39.31		
	k1	11.07	11.25	12.86	11.92		
	k2	13.78	13.92	12.67	12.87		
	k3	13.04	12.72	12.38	13.10		
	R	2.71	2.67	0.48	1.18		
Exopolymer	K1	2.499	2.808	2.532	2.862	The order for exopolymer: corn flour combined with 1 % glucose > > KH_2PO_4 > yeast extract > corn steep liquor	
	K2	3.537	3.087	3.429	3.087		
	K3	3.012	3.156	3.090	3.099		
	k1	0.833	0.936	0.844	0.954		
	k2	1.179	1.029	1.143	1.029		
	k3	1.004	1.052	1.030	1.033		
	R	0.346	0.116	0.299	0.079		

The yields of mycelia and exopolymer from *Hericium erinaceus* CZ-2 incubated in basal medium at various temperatures from 20 to 35 °C were investigated (data not shown). An optimum culture temperature of 25 °C was observed, and a very high and low temperature resulted in relatively lower yield of mycelial biomass. However, the most suitable temperature for high yield of exopolymer was 28 °C. These results indicate that the optimum culture temperatures for mycelia and exopolymer production were slightly different.

Effects of different carbon sources

Lots of reports showed that different carbon sources have different influences on the mycelia and exopolymer production by different mushroom strains (*17*,*18*). Therefore, effects of various carbon sources at the concentration of 2 % on the yields of mycelia and exopolymer production by *Hericium erinaceus* CZ-2 were examined. The results in Fig. 1 show that corn flour was the best carbon source for mycelial production (12.27 g/L), while glucose was chosen for exopolymer production

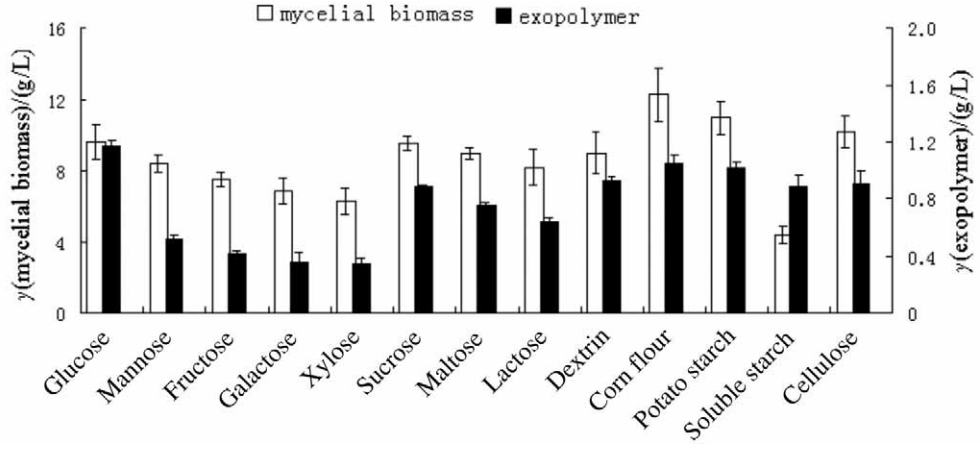

Fig. 1. Effect of carbon sources on mycelial biomass and exopolymer production by *H. erinaceus* CZ-2 in shake flask cultures

(1.17 g/L). This suggests that the strain *Hericium erinaceus* CZ-2 might secrete the powerful enzyme complexes which could decompose carbohydrates such as corn starch in the media, and thus provide the decomposed products to meet the carbon source demand for the strain growth. Otherwise, it is thought that corn starch is the best carbon source for fermentation industry due to its low cost and easily obtained material. References have revealed that the major sugar composition of exopolysaccharides was glucose (*19*,*20*). It is possible to facilitate the exopolysaccharide accumulation and secretion by supplementation of glucose. Hence, the suitable carbon source for the production of mycelial biomass and exopolymer was selected as corn flour combined with 1 % glucose.

The results in Fig. 2 indicate that the optimal concentration of corn flour for the maximum mycelial biomass and exopolymer production by the fungus *Hericium erinaceus* CZ-2 was 30 g/L. Under this condition, the yields of mycelial biomass and exopolymer reached 12.76 and 1.062 g/L, respectively.

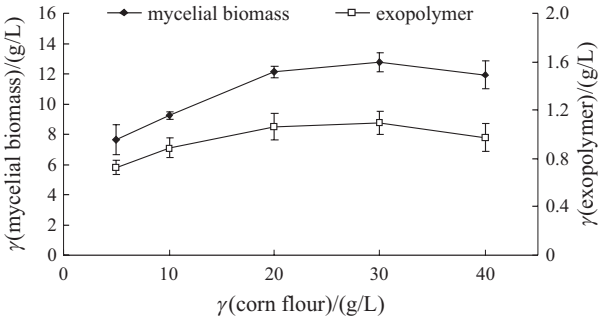

Fig. 2. Effect of initial concentrations of suitable carbon source (corn flour) on mycelial biomass and exopolymer production by *H. erinaceus* CZ-2 in shake flask cultures

Effects of different nitrogen sources

Fig. 3 shows the response levels when various nitrogen sources were added to the basal medium at a concentration of 2.0 g/L. Results indicate that the substitution of yeast extract in the medium with other nitrogen sources including organic nitrogen sources such as polypeptone, beef extract and bean cake powder could increase significantly the mycelia and exopolymer production. In contrast, the inorganic nitrogen sources, ammonium sulphate, ammonium nitrate, ammonium chloride and potassium nitrate, did not have very efficient effect on the response levels. Therefore, the yeast extract was selected as suitable nitrogen source for further investigation. Similar results that yeast extract contributed to the highest yield of the mycelia by *Cordyceps militaris* were also reported in other references (*21*).

The results of one-factor-at-a-time experiments showed that both mycelia (10.52 g/L) and exopolymer (0.982 g/L) production from *Hericium erinaceus* CZ-2 reached the highest level when the culture medium contained 4.0 g/L of yeast extract (Fig. 4). These phenomena may be due to the other organic nitrogen sources which also cooperated in nitrogen nutrition and influenced the yields of mycelia and exopolymer.

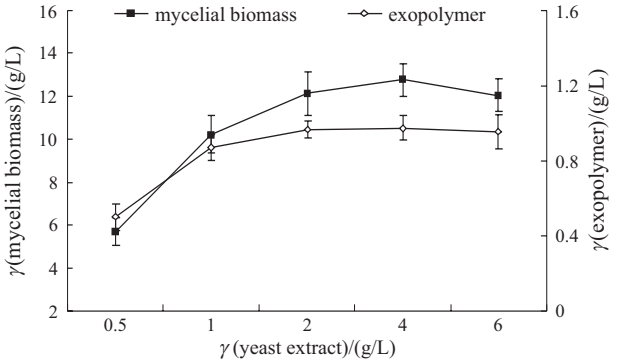

Fig. 4. Effect of initial concentrations of suitable nitrogen source (yeast extract) on mycelial biomass and exopolymer production by *H. erinaceus* CZ-2 in shake flask cultures

Effect of mineral elements and growth factors

The effect of growth factors and mineral elements on mycelial growth and exopolymer production are shown in Fig. 5. *Hericium erinaceus* CZ-2 was cultivated in basal medium containing various mineral elements (KH_2PO_4, $FeSO_4$, $MgSO_4{\cdot}7H_2O$, $ZnSO_4$, $CuSO_4$ and $CaCO_3$),

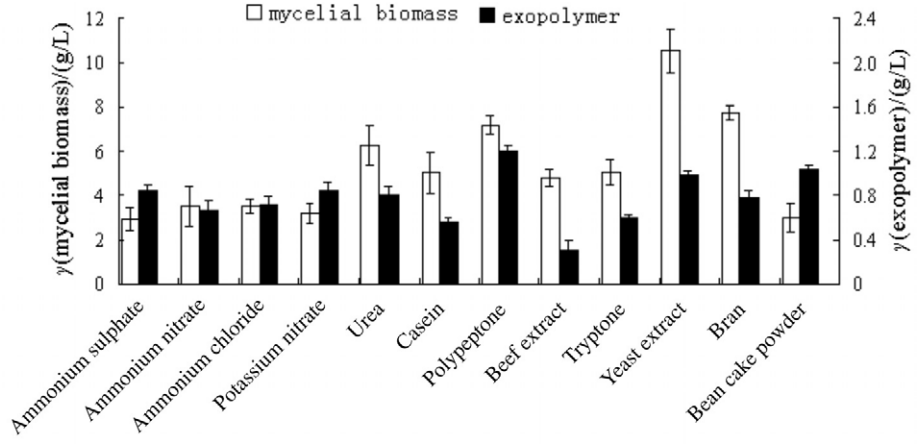

Fig. 3. Effect of nitrogen sources on mycelial biomass and exopolymer production by *H. erinaceus* CZ-2 in shake flask cultures

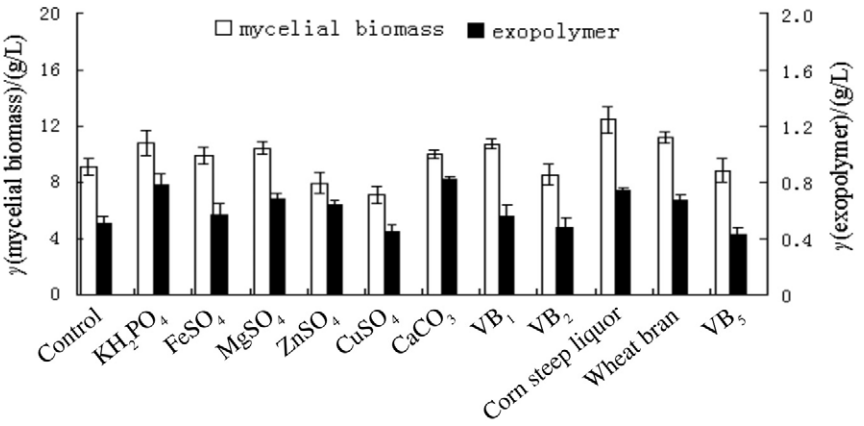

Fig. 5. Effect of minerals and cofactors on mycelial biomass and exopolymer production by *H. erinaceus* CZ-2 in shake flask cultures

and growth factors (VB_1, VB_2, VB_5, wheat bran and corn steep liquor) at a concentration of 1.0 g/L.

The mycelial biomass (10.79 g/L) and exopolymer production (0.818 g/L) were obtained by using KH_2PO_4 and $CaCO_3$, respectively. It has been reported that calcium and potassium ions are recognized as favourable mineral ions for mycelial and exopolysaccharide production in several mushroom fermentations (*22*).

Comparatively, the highest mycelial biomass (12.01 g/L) and exopolymer (0.737 g/L) production was achieved in culture medium containing corn steep liquor. Thus, corn steep liquor was the most efficient among the above five growth cofactors for production of mycelia and exopolymer by *Hericium erinaceus* CZ-2. Although corn steep liquor is regarded as one of the nitrogen sources (*23*), herein we took it as one of the growth cofactors because it contains sufficient nutritional compounds such as vitamins which could stimulate the growth of *H. erinaceus* CZ-2.

Orthogonal array method

The orthogonal array design technique is a traditional method that has been successfully applied to improve the culture media for fermentation processes and it provides the relationships among various factors, and the order of significant factors for the optimum results (*24–26*). In the present study, $L_9(3^4)$ design was applied to screen the significant factors according to the preliminary experiments. The four factors, carbon source (corn flour combined with 1 % glucose), the nitrogen source (yeast extract), growth factor (corn steep liquor) and mineral source (KH_2PO_4), and their relevant levels are shown in Table 1. In the course of optimization experiments, the fermentation temperature, initial pH, rotation speed and fermentation period were set at 25 °C, 5.5, 150 rpm and 7 days, respectively. The effects of these factors on mycelial yield and exopolymer production were calculated and the results of the analysis are shown in Table 2 and Fig. 6. The order of effects of factors on mycelial growth was: corn flour combined with 1 % glucose > yeast extract > corn steep liquor > KH_2PO_4; and the order of effects of factors on exopolymer production was: corn flour combined with 1 % glucose > KH_2PO_4 > yeast extract > corn steep liquor.

On the basis of the intuitive analysis, the optimization results were as follows: the maximal yield of mycelial biomass was obtained when the composition of the culture medium was (in g/L): corn flour 30, glucose 10, yeast extract 3, KH_2PO_4 1, $CaCO_3$ 0.5, and 15 mL/L of corn steep liquor; while the maximal exopolymer yield was achieved when the medium was composed of (in g/L): corn flour 30, glucose 10, yeast extract 5, KH_2PO_4 3, $CaCO_3$ 0.5, and 15 mL/L of corn steep liquor.

The validation experiments, done in triplicate, were conducted to obtain the maximum yields of mycelial biomass and exopolymer by *H. erinaceus* CZ-2 using the

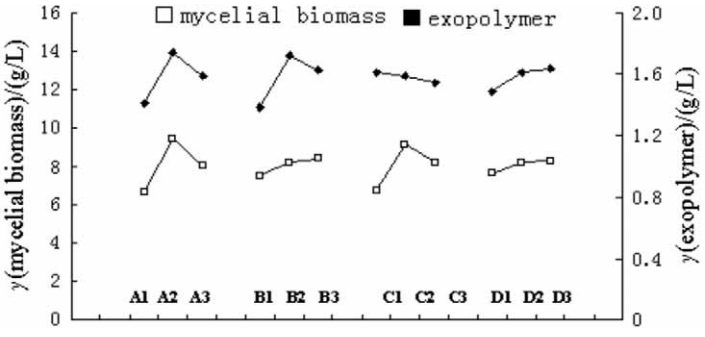

Fig. 6. Analysis of the relationship between medium composition and the yields of mycelial biomass and exopolymer by *H. erinaceus* CZ-2 in shake flask cultures
(A1–A3: corn flour 20, 30 and 40 g/L; B1–B3: yeast extract 1, 3 and 5 g/L; C1–C3: KH_2PO_4 1, 3 and 5 g/L; and D1–D3: corn steep liquor 5, 10 and 15 g/L, respectively)

above mentioned optimal culture compositions. The mean values of the mycelial biomass and exopolymer production were (16.07±0.45) and (1.314±0.071) g/L, much higher than those in basal culture media.

Batch fermentation

Fig. 7 shows the time course of 15-litre batch fermentation, including residual sugar concentration, dissolved oxygen (DO), pH, mycelial biomass, exopolymer production and pH value, recorded and analyzed during the whole fermentation process in the basal and optimized fermentation media.

The maximum mycelial biomass yield was 13.91 g/L in the basal medium after a 9-day cultivation. The values of pH, DO and residual glucose concentration of the culture broth decreased sharply from 5.5 to 3.7, 100 to 0.9 %, and 35 to 2 g/L, respectively. The logarithmic phase requiring plenty of nutrition and sufficient oxygen supplement appeared in the period from day 3 to day 8. Under the optimum medium, the maximum mycelial biomass was achieved at 20.53 g/L after a 9-day cultivation, while the concentration of exopolymer of 1.493 g/L was obtained, which were both higher than those in the basal medium. A sharp increase in pH was observed and pH value reached 2.7 at the end of the 9-day cultivation. The residual sugar concentration and DO values increased slightly at the end of the fermentation.

Conclusions

To date, although optimization of fermentation media and operating parameters has been applied for the submerged culture of various mushrooms, no reports are available in literature regarding the optimization of the medium composition for mycelial growth and exopolymer production by the screened strain *Hericium erinaceus* CZ-2. In this study, one-factor-at-a-time method combined with the orthogonal array design was applied to screen the suitable medium composition and operating parameters for the highest yields of the mycelia and exopolymer. Under optimal conditions, the values of the mycelial biomass and the exopolymer production yields were increased to 16.07 and 1.314 g/L, respectively. The batch fermentation results also showed that optimized

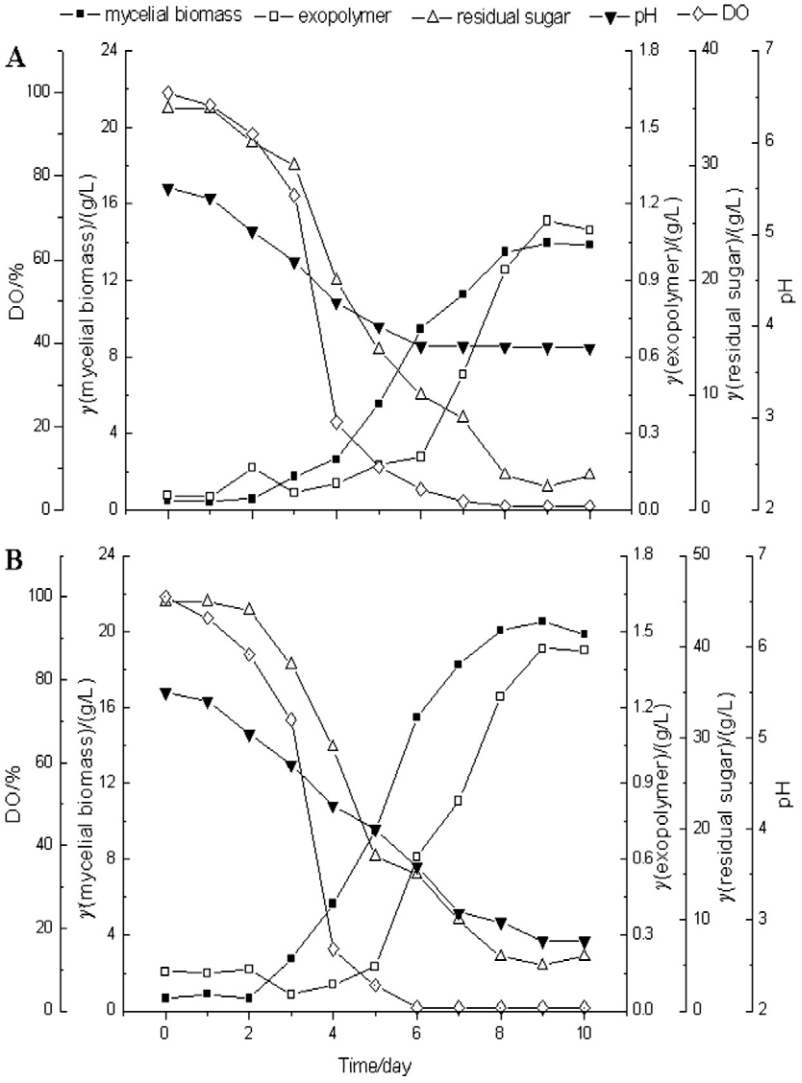

Fig. 7. Time profile of batch fermentation in 15-litre fermentor using the basal medium (A) and the optimized medium (B)

culture medium could improve the production of the mycelial biomass (20.53 g/L) and exopolymer (1.436 g/L) by *Hericium erinaceus* CZ-2 in a 15-litre fermentation process. Further investigation should emphasize the influence of the cultivation parameters in the 15-litre fermentation process for large scale production of mycelia and exopolymer by *Hericium erinaceus* CZ-2.

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