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SHORT COMMUNICATION

pH control strategy in a shaken minibioreactor for polysaccharide production by medicinal mushroom *Phellinus linteus* and its anti-hyperlipemia activity

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Abstract A process at various pH values ranging from 4.5 to 7.5 for production of mycelia and extracellular polysaccharide (EPS) by *P. linteus* fermentation in pH-controlled shaken bioreactor was investigated. A two-stage pH control strategy in which pH value was kept at 6.5 for the first 24 h, and then switched to 4.5 was developed successfully to enhance simultaneously the cell growth and EPS production. The maximum cell density and EPS production reached 15.13 ± 0.1 g/l on day 6 and 6.74 ± 0.1 g/l on day 4, respectively. The anti- hyperlipemia effect of EPS and intracellular polysaccharide (IPS) extracted from mycelia were observed that both EPS and IPS can obviously reduce the serum triglyceride (TG), the blood cholesterol (TC) and serum low density lipoprotein (LDL) level, and increase the high density lipoprotein (HDL) level of the hyperlipemia mice. Polysaccharides from submerged cultivation of medicinal fungus *P. linteus* have favorable potency to develop anti-hyperlipermia drugs.

Keywords Anti-hyperlipemia activity · EPS · IPS · *Phellinus linteus* · Shaken bioreactor

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Introduction

Phellinus linteus is one of the most well-know traditional medicinal mushrooms and has been widely used in Asian countries for years. *P. linteus* was known to be enriched in polysaccharides which have anti-tumor activity and other medicinal value. Many researchers have reported that polysaccharides isolated from *P. linteus* stimulated anti-tumor immunity, inhibited tumor growth and induced cytotoxic mechanism [1–3]. The methanolic extracts from the mycelial culture of *P. linteus* were also found having anti-hepatotoxins [4], and antioxidant activity [5]. However, it was little known that polysaccharides from submerged fermentation of *P. linteus* have anti-hyperlipemia activity.

In recent years, submerged fermentation of medicinal mushroom has received great interest in Asian countries as a promising alternative for efficient production of its valuable metabolites polysaccharide and other active products. There are many reports mentioning the various optimum submerged culture conditions of medicinal mushroom and their production of mycelial biomass and polysaccharide [6–9]. Within the various culture conditions, culture pH is one of the most important environmental factors in fungal fermentation, which can profoundly affect cell growth, product biosynthesis, mycelial morphology and broth rheology [10]. Submerged culture with pH control is an effective way to increase products of mycelia. In the submerged culture of medicinal mushroom *Ganoderma lucidum,* lee reported that shifting the pH from 3.0 to 6.0 at the initial phase of the exponential growth can improve cell growth and EPS production [11], Fang also reported that the initial pH varying within the range of 3.5–7.0 have a significant effect on the cell growth and polysaccharide biosynthesis of *G. lucidum* [12]. The initial pH 7.0 and 8.0

were best for polysaccharide production (EPS and IPS) and mycelial growth of medicinal mushroom *Lyophyllum decastes*, respectively [13]. The roughness and compactness of the pellets were affected by initial pH in the submerged culture of medicinal mushroom *Cordyceps militaris* [14]. It seems that different specials of medical mushrooms have obviously different pH control strategies for efficient production.

Recently, miniature bioreactors (MBRs) become an emerging field in which they have high-throughput capabilities and perform many cell cultivations in parallel [15, 16]. Shake flask is one of shaken bioreactors and usually used for multi-parameter parallel screening experiments, and culture optimization. In this paper, a novel shaken minibioreactor system which have low shear force and regulate pH automatically was designed to measure and control on-line pH, and employed to investigate the effect of pH on cell growth and extracellular polysaccharide (EPS) production of *P. linteus* fermentation, and a two-stage pH control strategy was developed to improve the productions of the mycelia biomass and EPS. Moreover, the anti-hyperlipemia activity of EPS and intracellular polysaccharide (IPS) extracted from mycelia have further been investigated in order to enlarge the medicinal value.

Materials and methods

Organism and medium

The strain of *P. linteus* was purchased from the collection bank of Huazhong Agricultural University (Hubei, China). The stock culture was maintained on potato-dextrose-agar slants. For Seed cultures, the medium composition was: glucose 20 g/l, yeast extracts 3 g/l, malt powder10 g/l. For fermentation, the medium consisted of sucrose 50 g/l, corn liquid 3 g/l, KH_2PO_4 10 g/l, $MgSO_4{\cdot}7H_2O$ 1 g/l, $CaCl_2{\cdot}$3 g/l and Vitamin $B_1$0.02 g/l.

Culture condition

The slants were inoculated with mycelia and incubated at 25 °C for 7 days, and then used for seed culture inoculation. The seed culture was grown in a 500 ml shake flask containing 100 ml of liquid medium and incubated at 25 °C on a rotary shaker (170 rpm) for 8 days. The fermentation cultivation was inoculated at 10%(v/v) of the above seed culture medium and kept at 25 °C and 160 rpm.

pH controlled cultivation in shaken minibioreactor

The shaken minibioreactor system was designed by Shanghai Guoqiang Bioengineering Equipment Co., Ltd China. Specially parallel 650 ml shaken bioreactors made of glass are used with a vertical port for the pH-probe. The intermittent feeding system was applied for parallel pH automatic controlled by base or acid addition. (See Fig. 1). The culture temperature and the inoculums size were the same as in shake flasks. Samples were taken every 24 h for the analyses of dry cell weight, EPS production, total sugar and residual sugar concentration. The shaken minibioreactor fermentation under each pH value ranging from 4.5 to 7.5 was repeated, and the data shown represent the mean values with the standard deviations.

The anti-hyperlipemia activity of EPS and IPS

For the determination of anti-hyperlipemia activity of IPS extracted from mycelia and EPS. Female SD mice (120–150 g, purchased from Laboratory Animal Facility of Third Military Medical University, China) were used, They were housed in a standard environmental condition and fed with high fat diets, contain vitelline flour 20%, lard 10%, bile salt pig 1% to induce hyperlipemia. After 3 weeks, animals were selected based on their body weight and allocated randomly to the following groups: hyperlipermia control, groups treated with 100, and 200 mg/kg per day of EPS, groups treated with 100, and 200 mg/kg per day of IPS and masculine groups treated with 60 mg/kg per day of Chinese drug products of Jiangzhiling (Chongqing Tongjunge Co. Ltd, China). All groups were treated once a day for 15 days. At the end of treatment liver weights were detected and regarded as absolute weight. Serum Low density lipoprotein (LDL), high density lipoprotein (HDL), triglyceride (TG) and the blood cholesterol (TC) were detected with an automated blood analyzer.

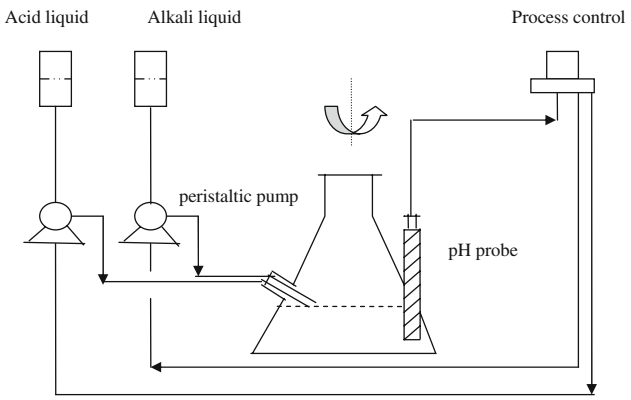

Fig. 1 A schematic diagram of the 650 ml shaken bioreactor with pH controlled on-line by feeding hydrochloric acid or sodium hydroxide

Determination of dry weight, residual sugar and total sugar

Dry cell weight (DW) was obtained by centrifugation (4,000×*g*, 10 min), washing the cells for three times with distilled water, and drying at 60 °C to a constant weight. The residual sugar was assayed by the dinitrosalicylic acid method (DNS method)[17] and total sugar was tested by phenol-sulfuric acid method [18].

Measurement of extracellular and intracellular polysaccharides

For the determination of EPS, after removal of mycelia by centrifugation (4,000×*g*, 10 min), the supernatant were concentrated at 60 °C to one-third of its total volume. The concentrated liquor was first precipitated with addition of 95% (v/v) ethanol by 30% (v/v), stored at 4 °C for 24 h and then separated by centrifugation at 4 °C (8,000×*g*, 15 min). The precipitate was discarded and the resulting supernatant was further mixed with addition of 95% (v/v) ethanol by 70% (v/v). Precipitation of EPS proceeded at 4 °C for 24 h and collected by centrifugation at 8,000×*g* for 15 min, discarding the supernatant. The precipitation of EPS was lyophilized and the weight of it was estimated [19].

For the determination of IPS, the dried mycelia were extracted by using hot water at 90 °C (2 h), and the supernatants were treatment with the method of EPS.

Results and discussion

Cell growth and EPS production at different pH control levels

In order to investigate the effect of pH control on mycelia growth and EPS biosynthesis, batch experiments were performed in shaken minibioreactor by varying pH value from 4.5 to 7.5. Profiles of residual sugar and total sugar concentration are shown in Fig. 2a and b. The consumption of total sugar obviously speeded up at pH 4.5 and produced more residual sugar for cell growth. Figure 2c and d shows the time profiles of cell growth and EPS production at different pH control levels. The results indicated that the maximum cell density at the pH value of 7.5, 6.5, 5.5, 4.5 was 4.67 ± 0.05, 14.43 ± 0.1, 14.75 ± 1.58, and 10.74 ± 0.42 g/l on day 4, 2, 3, and 3, respectively, while the maximum EPS production titer at the pH value of 7.5, 6.5, 5.5, 4.5 was 1.45 ± 0.06, 1.75 ± 0.05, 1.67 ± 0.14, and 6.8 ± 0.1 g/l on day 4, 4, 6, and 2, respectively. Kinetic parameters with batch fermentation under different pH control value were shown in Table 1. The growth rate of cell biomass at pH 6.5 was obviously quick than that of the other pH value, while the production formation rate of EPS at pH 4.5 was significantly improved compared with the other pH value. The above results indicated that the cell growth and EPS production were differently regulated under pH control

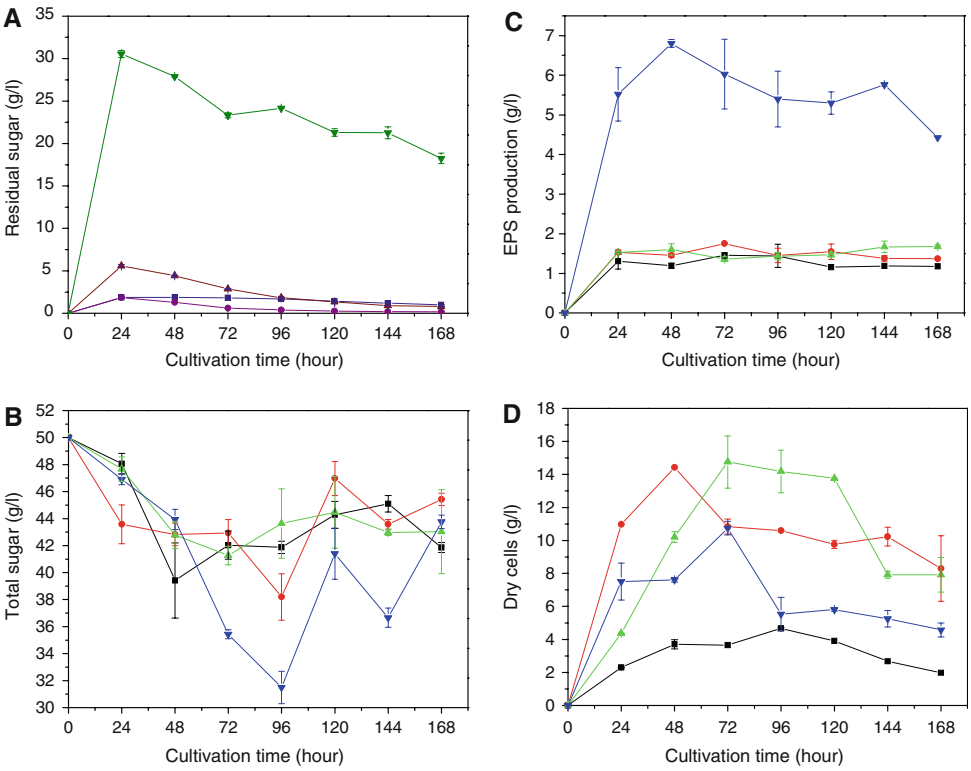

Fig. 2 Time courses of residual sugar consumption (**a**), total sugar (**b**), EPS production (**c**), and cell growth (**d**), under different pH control value. Symbols for pH value 7.5 (*square*), 6.5 (*circle*), 5.5 (*triangle*) and 4.5 (*inverted triangle*). The *error bars* in the figure indicate the standard derivations from two independent samples

Table 1 Comparison of kinetic parameters with batch fermentation under different pH control levels

Parameters	pH control levels			
	7.5	6.5	5.5	4.5
Maximum dry cell weight (g/l)	4.67 ± 0.05 (day 4)[a]	14.43 ± 0.1 (day 2)	14.75 ± 1.58 (day 3)	10.74 ± 0.42 (day 3)
Maximum EPS concentration (g/l)	1.45 ± 0.06 (day 4)	1.75 ± 0.05 (day 4)	1.60 ± 0.14 (day 6)	6.80 ± 0.1 (day 2)
Cell yield on sugar (g DW/g sugar)	0.09 ± 0.001	0.29 ± 0.002	0.30 ± 0.032	0.21 ± 0.008
EPS yield on sugar (mg/g sugar)	0.03 ± 0.001	0.04 ± 0.001	0.03 ± 0.003	0.13 ± 0.002
EPS productivity (g/l per day)	0.48 ± 0.02	0.58 ± 0.02	0.27 ± 0.07	3.4 ± 0.05

[a] Culture time when the maximum biomass was reached

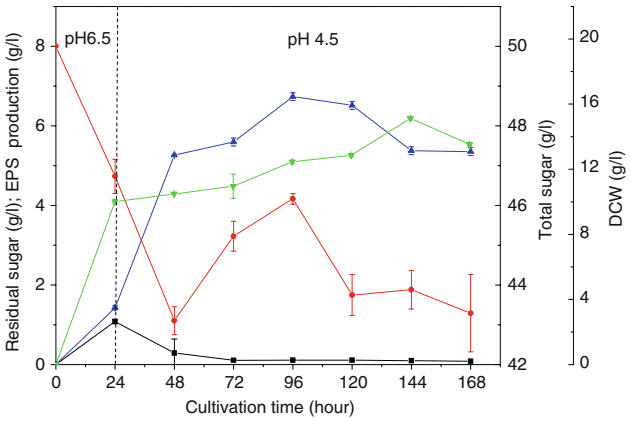

Fig. 3 Time profiles of cell growth and EPS production by using a two-stage pH control strategy. Residual sugar (*square*), total sugar (*circle*), cell growth (*inverted triangle*) and EPS production (*triangle*). The pH value was kept at 6.5 in the first 24 h then switched to 4.5 by on-line control

levels. During the submerged fermentation of medicinal mushroom, Fang [12] also reported that lowering the initial pH from 6.5 to 3.5 gradually led to a higher production of extracelluar polysaccharide and a higher specific production of IPS.

Optimal pH-shift strategy for cell growth and EPS production

Based on the above batch experiments with pH control, an optimal pH-shift strategy was performed, in which the pH value was kept at 6.5 for the first 24 h, and then switched to 4.5 to enhance simultaneously the productions of mycelial biomass and EPS. The results were shown in Fig. 3. The maximum cell density and EPS production was 15.13 ± 0.1 g/l on day 6 and 6.74 ± 0.1 g/l on day 4, respectively. It is clear that, by adopting a simple and very effective strategy of pH-shift batch fermentation in the shaken minibioreactor, the cell mass and EPS production were enhanced simultaneously. The results obtained in this study were very useful for the simultaneous, highly efficient production of the biomass, and polysaccharide on a large scale.

The anti-hyperlipemia activity of EPS and IPS

The effect of EPS and IPS extracted from mycelia of medicinal mushroom *P. linteus* on the hyperlipemia activity is shown in Table 2. Compared with hyperlipemia control, all of high and low dose groups of EPS and IPS have can obviously reduce the serum TG, TC and LDL level and increase the serum HDL level of the hyperlipemia mice. Table 2 clearly showed that the decreasing rate of TG, TC and LDL level and increasing rate of HDL of high dose groups of EPS were higher than those of low dose groups, while the dose effects of IPS have no coincidence with the decreasing rate of TG and the increasing rate of HDL. Compared with masculine control, both EPS and IPS have similar anti-hyperlipemia activity. These results indicated that polysaccharides including EPS and IPS from submerged cultivation by medicinal mushroom *P. linteus* can significantly reduce the blood total cholesterol levels,

Table 2 Effects of EPS an IPS on contents of TG, TC, HDL and LDL in experimental hypercholesterolemia mice

Groups	TG (mmol/L)	TC (mmol/L)	HDL (mmol/L)	LDL (mmol/L)
Normal control	0.690 ± 0.44	1.690 ± 0.300	0.610 ± 0.073	1.080 ± 0.223
Masculine control	1.211 ± 1.345	2.395 ± 0.275***	0.749 ± 0.180	1.646 ± 0.895***
Hyperlipemic control	1.573 ± 0.887	3.489 ± 0.434	0.710 ± 0.166	2.625 ± 0.219
Low dose of IPS	0.99 ± 0.670**	2.480 ± 0.820***	0.843 ± 0.137	1.589 ± 0.910***
High dose of IPS	1.190 ± 0.650*	1.924 ± 0.166***	0.819 ± 0.131	1.105 ± 0.165***
Low dose of EPS	1.168 ± 0.782*	2.776 ± 0.135***	0.802 ± 0.25	1.974 ± 0.516***
High dose of EPS	0.868 ± 0.592***	2.073 ± 0.477***	0.813 ± 0.277	1.26 ± 0.360***

Compared with masculine control *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

which can used as an alternative source to product anti-hyperlipermia drugs.

Conclusion

In this work, a novel pH control strategy based on a shaken minibioreactor system for efficient production of mycelia and polysaccharide by medicinal mushroom *P. linteus* was developed. A two-stage pH-shift strategy in which pH value was kept at 6.5 for the first 24 h, and then switched to 4.5 was developed successfully to enhance simultaneously cell growth and polysaccharide production. The fundamental information obtained in this work is beneficial for further development of *P. linteus* submerged cultivation process on a large scale. Moreover, it was interesting that polysaccharides obtained from submerged cultivation of *P. linteus* have favorable potency to develop anti-hyperlipermia drugs.

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