Efficient Production of Exopolysaccharide by Submerged Fermentation of *Hypsizygus marmoreus* Using a Two-stage pH Control Strategy

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The effects of culture conditions and pH values on the exopolysaccharides (EPS) production by *Hypsizygus marmoreus* were investigated in this work. The results showed that the optimal carbon and nitrogen sources were glucose and yeast extract at concentrations of 30 g L^{-1} and 12.5 g L^{-1}, respectively. In constant pH fermentation, high biomass concentration and the specific cell growth rate were achieved at pH 6.5, while high EPS concentration and the specific EPS production rate were obtained at pH 7.5, respectively. Based on kinetics analysis, a two-stage pH strategy was proposed in which pH was controlled at 6.5 to maintain high cell growth and glucose consumption in the first 48 h, and then shifted to 7.5 for increasing EPS production. The maximal EPS concentration reached 2.31 ± 0.07 g L^{-1} by applying this strategy. The EPS productivity (0.024 g L^{-1} h^{-1}) increased 58.94 %, 172.7 % and 35.73 %, respectively, compared with that of the natural pH, and controlling pH at 6.5 and 7.5.

Keywords

Hypsizygus marmoreus, exopolysaccharide, submerged fermentation, kinetics analysis, two-stage pH strategy

Introduction

Mushrooms have long been used as a food and medical resource. Modern scientific studies demonstrate that mushrooms are an abundant source of a wide range of bioactive natural products. Polysaccharides are the best-known mushroom-derived substances, and have various biological activities, such as antitumor, immunomodulatory, antihyperlipidemic, hepatoprotective, antioxidative and anti-fatigue effects[1–5].

Polysaccharides from medicinal mushrooms such as *Ganoderma lucidum*, *G. tsugae*, *G. applanatum*, *Cordyceps sinensis*, *C. militaris* have been studied extensively[6–10], and the research for new bioactive polysaccharide has been extended to other mushrooms. *Hypsizygus marmoreus* is a popular edible mushroom containing a variety of bioactive substances, such as polysaccharides, proteins, trace elements, essential amino acids, lectins, terpenoids, tocopherols, ascorbic acid, enzymes and dietary fiber[11], and has been considered as a functional food in East Asia because of its antifungal, antiproliferative, immunomodulating, antioxidant and hepatoprotective activities[1,11–13].

Generally, polysaccharide could be extracted from the fruiting body of mushrooms. However, this is time-consuming, low-productive and economically unviable, and the production rate is insufficient to meet the current market demand. Submerged culture has been a fast alternative method to produce medicinal mushroom mycelia and bioactive compounds, and a large amount of studies have been focusing on the polysaccharide production by growing mushroom mycelium in liquid culture with defined nutrients[6–8,14,15]. A vast range of valuable polysaccharide has been extensively exploited from medicinal mushrooms by submerged culture, such as *G. lucidum*, *Grifola frondosa*, *C. militaris*[6–9,16]. A few works on polysaccharide production have also been carried out by submerged culture of *H. marmoreus*[17].

To improve polysaccharide production, some techniques such as media optimization[16,17], repeated-batch fermentation[6–8], pH-shift, and dissolved-oxygen transfer (DOT)-shift integrated fed-batch fermentation[14], multi-fed batch[18] have been performed on the submerged culture of medicinal mushrooms. Mycelial growth and metabolism can be significant-

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ly affected by media composition and other chemical and physical factors in submerged culture, and pH is one of the vital parameters because it can influence the solubility of salts, the ionic state of substrates, cell membrane function, and metabolite biosynthesis[19]. Various pH conditions were studied and two-stage pH strategy had been utilized to enhance the mycelial growth and product biosynthesis in some fungi fermentation[14,15,19–21]. The polysaccharide productivity of *Auricularia auricula* increased by 93.3 % when culture pH was controlled at 5.0 in the first 48 h and then switched to 5.5 compared with that of non-pH control[15]. The maximum concentration of rhamsan gum was 12.83 % higher when the culture pH of *Sphingomonas* sp. was controlled at 7.5 during the first 10 h and then shifted to 7.0, than that at natural pH[19]. Investigation suggested that lower pH supported cell growth of *Aerobasidium pullulans* while higher pH stimulated pullulan synthesis, and maximum pullulan production could be conducted when two-stage pH strategy was performed[20]. Tang *et al.* suggested that the pH-shift from 3.0 to 4.5 on day 4 was beneficial to the intracellular polysaccharide production in the cultivation of *G. lucidum* CGMCC 5.616, and Lee *et al.* reported that the pH-shift from 3.0 to 6.0 on day 2 was best for extracellular polysaccharide biosynthesis in the fermentation of *G. lucidum* ASI 7004[14,21].

In this work, the effect of various pH on polysaccharide production of *H. marmoreus* was systematically investigated. A pH control strategy was proposed to achieve a high concentration and high productivity of polysaccharide in a 5.0 L fermenter based on kinetic parameters analysis. A two-stage pH control strategy was established to improve the polysaccharide production and the effectiveness was verified experimentally.

Materials and methods

Microorganism

The strain of *H. marmoreus* WZ019 was obtained from Sanming Institute of Fungi (Sanming, People's Republic of China). The fungus was maintained on potato dextrose agar (PDA) slants at 4 ºC and subcultured every 2 weeks.

Culture media and cultivation conditions

The inocula were prepared in 500-mL Erlenmeyer flasks containing 150-mL media at 27 °C for 6 days with shaking at 170 rpm. The culture medium contained (g L^{-1}): glucose 30, yeast extract 10, corn powder 10, $MgSO_4$ 0.75, K_2HPO_4 1.5.

For carbon and nitrogen sources screening, the inocula were inoculated at 10 % (v/v) into 500 mL Erlenmeyer flasks containing 150 mL media, and flask cultivation was carried out at 27 °C for 7 days with shaking at 170 rpm. The basal fermentation media consisted of (g L^{-1}): glucose 20, yeast extract 10, $MgSO_4$ 0.75, K_2HPO_4 1.5.

For various pH investigations, fermentations were carried out in a 5-L fermenter (Winpact FS-02VD, USA) with 4.0 L media. The agitation speed and aeration rate were controlled at 250 rpm and 2 vvm, respectively, pH was controlled at a fixed level in the range of 4.5–8.5 with addition of either 1 mol L^{-1} NaOH or 1 mol L^{-1} HCl.

Analytical methods

Collected samples were centrifuged at 3000 rpm for 15 min, and the mycelia were collected and washed twice with distilled water. The biomass was weighed after gradual drying at 65 ºC and 105 ºC until a constant weight, and cooled in a desiccator as described by Wan-Mohtar *et al.*[6] The supernatant was used for polysaccharide measurement.

The extracellular polysaccharide (EPS) produced by *H. marmoreus* was precipitated by addition of 4 volumes of 95 % (v/v) ethanol, and then left overnight at 4 ºC. The precipitated polysaccharide was collected by centrifugation at 4,000 rpm for 20 min, and washed with 80 % (v/v) ethanol three times. The polysaccharide content was determined by phenol-sulfuric acid method[22]. Residual glucose concentration was measured by 3,5-dinitrosalicylic acid (DNS) method using glucose as a standard[23].

Kinetic parameters calculation

The kinetic parameters were calculated as described by Wan-Mohtar *et al.*[8] The specific cell growth rate (μ, h^{-1}), the specific glucose consumption rate (q_s h^{-1}), and the specific EPS production rate (q_p, h^{-1}) were estimated from the experimental or fitted data of biomass (X, g L^{-1}), residual glucose concentration (S, g L^{-1}), and EPS concentration (P, g L^{-1}). The cell yield on glucose ($Y_{X/S}$) and the EPS yield on glucose ($Y_{P/S}$) were calculated from μ, q_s, and q_p. The parameters were respectively calculated by the following definitions, and the calculations were implemented with Microsoft Excel program. Kinetic parameters were subjected to ANOVA by Bonferroni's Post Test using STATISTICA 6.0, and differences at $p < 0.05$ were considered significant.

$$\mu = \frac{1}{X}\frac{\mathrm{d}X}{\mathrm{d}t} = \frac{1}{X}\lim\frac{\Delta X}{\Delta t} \tag{1}$$

$$q_s = -\frac{1}{X}\frac{\mathrm{d}S}{\mathrm{d}t} = -\frac{1}{X}\lim\frac{\Delta S}{\Delta t} \tag{2}$$

$$q_p = \frac{1}{X}\frac{dP}{dt} = \frac{1}{X}\lim\frac{\Delta P}{\Delta t} \quad (3)$$

$$Y_{X/S} = -\frac{dX}{dS} = \frac{\mu}{q_s} \quad (4)$$

$$Y_{P/S} = -\frac{dX}{dS} = \frac{q_p}{q_s} \quad (5)$$

Results and discussion

Effect of carbon and nitrogen on the growth and EPS production

Carbon and nitrogen sources and their concentrations generally play a significant role because these nutrients are directly linked with cell proliferation and metabolite biosynthesis[16]. To identify the best carbon sources for mycelial growth and EPS production, different carbohydrates, i.e., glucose, lactose, xylose, sucrose, fructose, galactose, were tested. As shown in Fig. 1a, glucose was the optimal carbon source for the fermentation of *H. marmoreus*, which was in accordance with the results of Wang *et al.*[17] Mycelial growth increased with an increase in initial glucose concentrations between 5 and 40 g L^{-1}, whereas the EPS production enhanced insignificantly when the initial glucose concentration was above 30 g L^{-1} (Fig. 1b).

To investigate the effect of nitrogen sources on mycelia growth and polysaccharide production of *H. marmoreus*, different nitrogen sources (soybean powder, peptone, yeast extract, ammonium sulfate and ammonium nitrate) were examined, and results showed that yeast extract was the optimal nitrogen source at 12.5 g L^{-1} (Figs. 1c–1d).

Time course of polysaccharide fermentation without pH control

Culture conditions, such as carbon source, nitrogen source, pH, and temperature varies significantly from species to species. Moreover, the efficiency of any biosystem is strictly pH-dependent owing to strong dependence on enzymatic activity and cellular metabolism[24]. Previous research has shown that the preferable initial pH for cell growth and polysaccharide production by *H. marmoreus* ZJ-029 was 6.5–7[17]. Fig. 2 shows the time courses of biomass formation and EPS production by *H. marmoreus* in a 5-L fermenter without pH control. It was observed that the pH of culture broth changed from initial 6.5 to 5.14 during the fermentation, and the main stage of pH decrease was 24–96 h, which was the exponential phase of *H. marmoreus*. Before the pH decreased to 5.4, the cell growth increased rapidly and reached the maximum of 8.50 g L^{-1} at

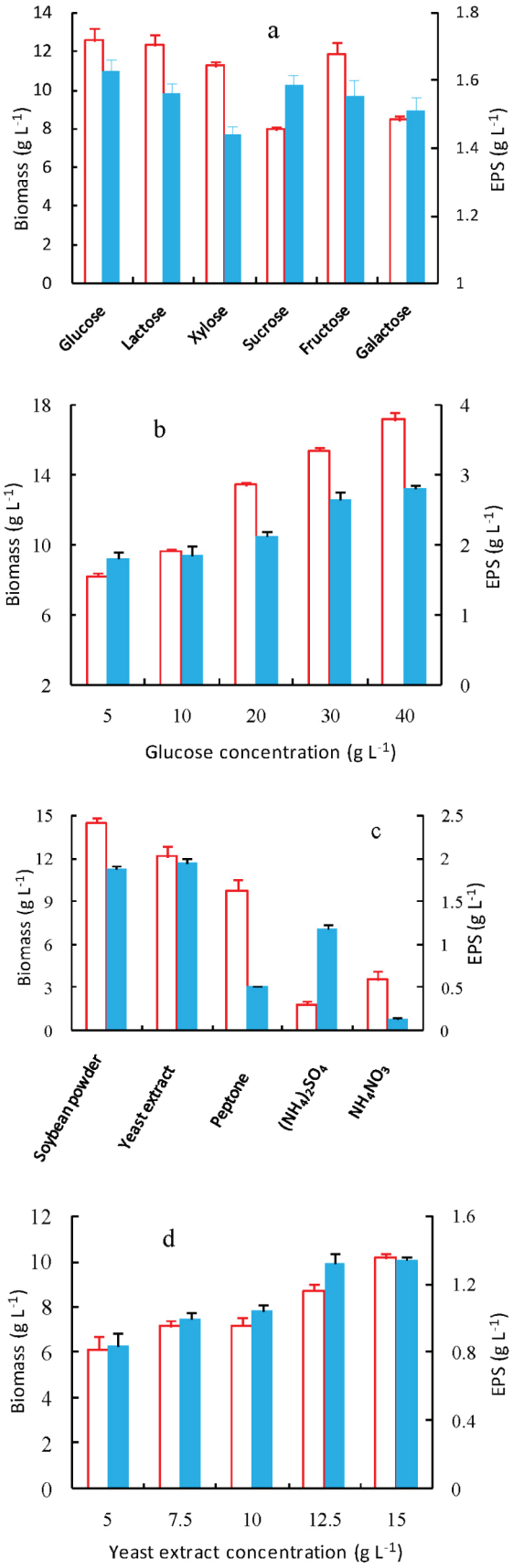

Fig. 1 – *Effects of carbon sources (a), glucose concentration (b), nitrogen sources (c) and yeast extract concentration (d) on mycelial growth and EPS biosynthesis by H. marmoreus. EPS (■) and biomass (□).*

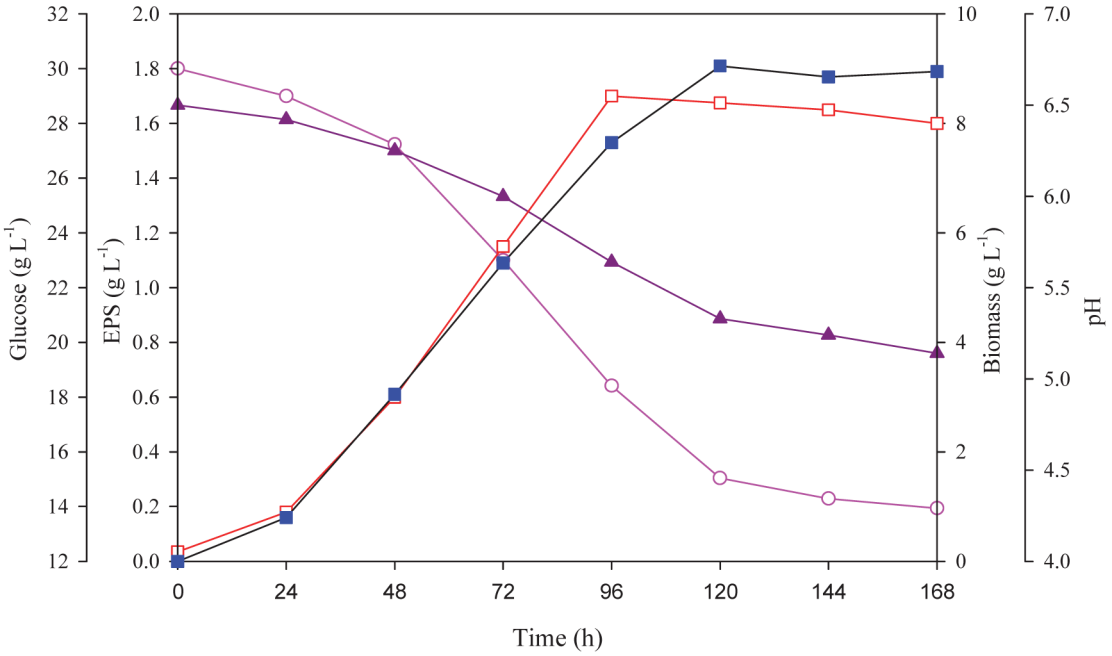

Fig. 2 – *Time courses of submerged culture of H. marmoreus in 5-L fermenter under non-pH control. EPS (■), biomass (□), pH (▲), and glucose (○).*

96 h of cultivation. After that, the mycelial formation reduced and the biomass concentration decreased slowly. Polysaccharide was produced from 48 h and the highest concentration reached 1.81 g L^{-1} at 120 h. This indicated that the EPS production was partially correlative to the mycelia growth by *H. marmoreus*. The glucose consumption trend was in accordance with the cell proliferation and polysaccharide biosynthesis, and the concentration of residual glucose in the fermentation broth reduced sharply from 24 h to 120 h. The glucose consumption rate decreased after 120 h, and high residual glucose concentration (15.0 ± 0.43 g L^{-1}) was detected at the end of fermentation. Fig. 2 indicates that the pH in the culture broth was critical in the fermentation of *H. marmoreus*, and the cell growth could be prolonged and the polysaccharide production could be improved by controlling the pH in the culture broth. Previous researches have shown that high pH was favorable for some polysaccharide production, such as pullulan[20], *Ganoderma* polysaccharide[21]. Similarly, EPS of *H. marmoreus* was biosynthesized in higher pH level.

Time courses of polysaccharide fermentation at different pH

The pH of fermentation media can influence the morphology of some microbial strains, which may subsequently influence cell growth and metabolite production[20,21]. To explore the effects of controlled pH on the glucose consumption, mycelia growth and EPS production of *H. marmoreus*, the pH was respectively maintained at pH 4.5, 5.5, 6.5, and 7.5 during the cultivation. As shown in Fig. 3, high pH was beneficial to mycelial growth and EPS production. The biomass increased when pH was increased from 4.5 to 6.5, and the maximum value (10.6 ± 0.36 g L^{-1}) was detected at pH 6.5 after 144 h (Fig. 3a). Consistent with the increase in cell growth, the glucose consumption increased from pH 4.5 to 6.5 (Fig. 3b). However, the desired pH for polysaccharide biosynthesis and mycelia growth was inconsistent, and the maximum EPS concentration (2.08 ± 0.07 g L^{-1}) was achieved at pH 7.5 after 120 h (Fig. 3c).

Kinetics analysis of EPS production at different pH

To enhance the EPS concentration and productivity, kinetic parameters of EPS production at different pH, μ, q_s, and q_p were calculated based on the data in Figs. 3a–3c. As shown in Figs. 3d–3f, the patterns of mycelial growth, glucose consumption, and EPS production under different pH were significantly different. The maximal mycelial growth rate (μ) of different constant pH was all at 24 h and the values were insignificant (p>0.05) at pH 5.5, pH 6.5 and pH 7.5. However, the mycelia growth rate at pH 6.5 was significantly higher than that at other pHs at 48 h (Table 1). The maximal specific cell growth rate and maximal specific glucose consumption rate were respectively observed at pH 6.5 from 24 h to 48 h (Figs. 3d and 3e), and the highest cell yield on glucose was achieved at pH 6.5 (Fig. 4a). The pH values did not affect specific EPS produc-

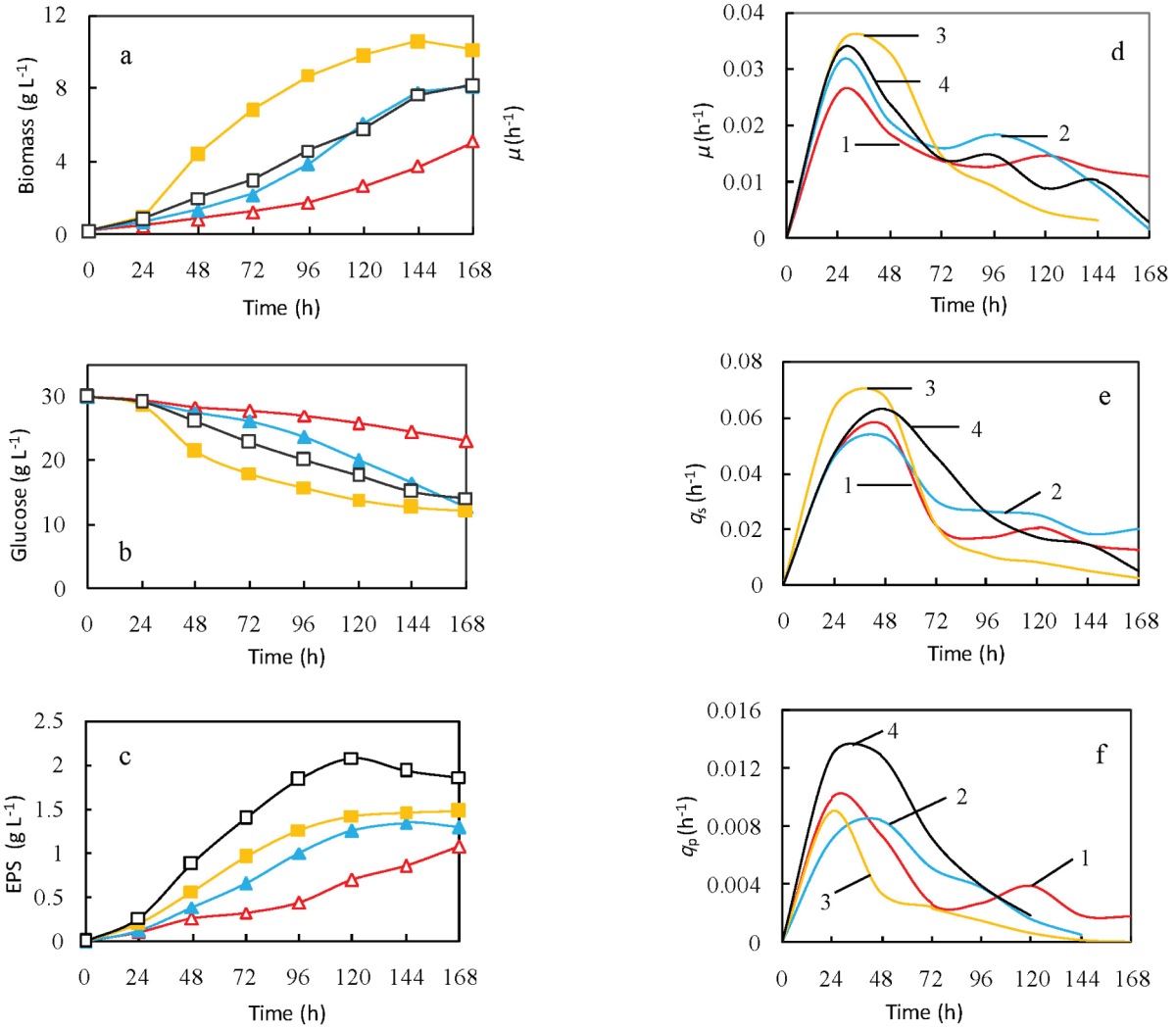

Fig. 3 – *Time courses of submerged culture of H. marmoreus in 5-L fermenter under different constant pH. pH 4.5 (△ and 1), pH 5.5 (▲ and 2), pH 6.5 (■ and 3) and pH 7.5 (□ and 4).*

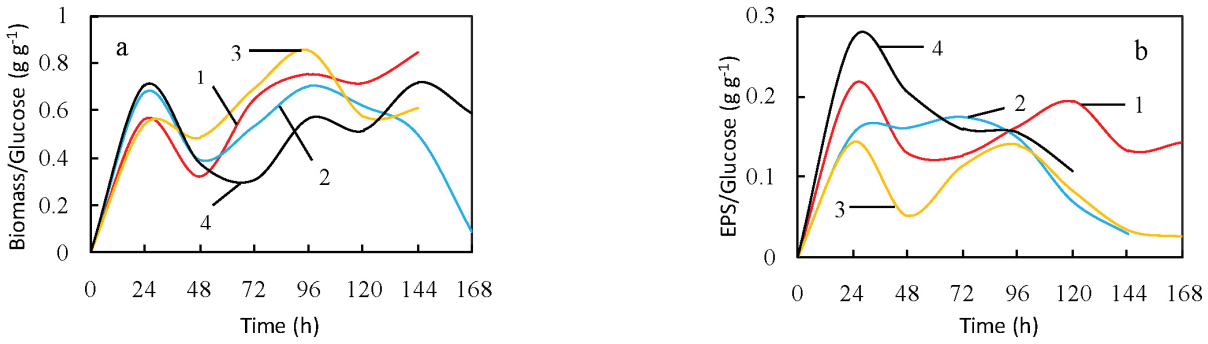

Fig. 4 – *Changes in biomass and EPS yields on glucose at different constant pH. pH 4.5 (1), pH 5.5 (2), pH 6.5 (3) and pH 7.5 (4).*

tion rate (q_p) at the initial stage (0–24 h). The maximal EPS production rate was achieved at pH 7.5 from 24 h to 48 h, and the EPS yield on glucose ($Y_{P/S}$) was obtained at pH 7.5 (Figs. 3f and 4b). The concentrations of EPS at pH 7.5 (2.08 ± 0.07 g L^{-1}) increased by 14.92 % compared with that of the natural pH (1.81 ± 0.03 g L^{-1}).

Based on the analysis of μ, q_s, and q_p, it was appropriate to control pH at 6.5 to maximize the mycelial growth rate and maintain high specific glucose consumption rate in the first 48 h, and then shift pH to 7.5 to maintain high specific EPS production rate (q_p) in the submerged culture of *H. marmoreus*. The pH variation obtained in this study

Table 1 – *Time course of kinetic parameters on the submerged culture of H. marmoreus in 5-L fermenter under different constant pH*[a]

Time (h)	μ				q_s				q_p			
	pH 4.5	pH 5.5	pH 6.5	pH 7.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5	pH 4.5	pH 5.5	pH 6.5	pH 7.5
0	0	0	0	0	0	0	0	0	0	0	0	0
24	0.0259 ± 0.0013b	0.0311 ± 0.0014a	0.0342 ± 0.0013a	0.0335 ± 0.0003a	0.0483 ± 0.0116	0.0397 ± 0.0135	0.0589 ± 0.0063	0.0430 ± 0.0207	0.0099 ± 0.0004b	0.0071 ± 0.0007c	0.0090 ± 0.0007b	0.0128 ± 0.0003a
48	0.0183 ± 0.0009d	0.0204 ± 0.0001c	0.0327 ± 0.0002a	0.0237 ± 0.0004b	0.0545 ± 0.0358	0.0532 ± 0.0200	0.0673 ± 0.0059	0.0629 ± 0.0034	0.0074 ± 0.0008b	0.0084 ± 0.0002b	0.0034 ± 0.0000c	0.0129 ± 0.0004a
72	0.0137 ± 0.0001	0.0159 ± 0.0015	0.0147 ± 0.0009	0.0139 ± 0.0016	0.0213 ± 0.0170	0.0302 ± 0.0109	0.0215 ± 0.0067	0.0459 ± 0.0043	0.0027 ± 0.0007c	0.0052 ± 0.0002b	0.0024 ± 0.0001c	0.0073 ± 0.0008a
96	0.0127 ± 0.0011b	0.0184 ± 0.0003a	0.0091 ± 0.0003c	0.0146 ± 0.0007b	0.0168 ± 0.0038b	0.0263 ± 0.0026a	0.0106 ± 0.0021b	0.0258 ± 0.0019a	0.0028 ± 0.0001b	0.0038 ± 0.0003a	0.0015 ± 0.0001c	0.0040 ± 0.0002a
120	0.0145 ± 0.0005a	0.0152 ± 0.0002a	0.0047 ± 0.0009b	0.0088 ± 0.0009c	0.0207 ± 0.0193	0.0249 ± 0.0029	0.0081 ± 0.0020	0.0170 ± 0.0020	0.0039 ± 0.0001a	0.0017 ± 0.0000b	0.0007 ± 0.0000c	0.0018 ± 0.0001b
144	0.0120 ± 0.0003a	0.0089 ± 0.0010b	0.0031 ± 0.0000c	0.0100 ± 0.0012ab	0.0143 ± 0.0051ab	0.0181 ± 0.0041a	0.0052 ± 0.0018b	0.0140 ± 0.0031ab	0.0019 ± 0.0003a	0.0005 ± 0.0000b	0.0002 ± 0.0001b	–0.0008 ± 0.0001c
168	0.0109 ± 0.0012a	0.0017 ± 0.0008b	–0.0021 ± 0.0002c	0.0028 ± 0.0013b	0.0125 ± 0.0099ab	0.0199 ± 0.0031a	0.0021 ± 0.0019b	0.0047 ± 0.0053ab	0.0018 ± 0.0001a	–0.0003 ± 0.0001c	0.0000 ± 0.0001b	–0.0004 ± 0.0001c

[a]The variance of kinetics parameters (μ, q_s, q_p) at different pH was respectively analyzed and the different letters were significantly different at $p < 0.05$.

Table 2 – *Kinetic parameters of different pH strategy by H. marmoreus in a 5-L bioreactor*

Parameters	Natural pH	pH 4.5	pH 5.5	pH 6.5	pH 7.5	Two-stage pH strategy
Biomass (g L^{-1})	8.50 ± 0.32	5.05 ± 0.29	8.07 ± 0.48	10.6 ± 0.36	8.20 ± 0.51	12.3 ± 0.36
Time of maximal of EPS achieved (h)	120	168	144	168	120	96
Concentration of maximal EPS (g L^{-1})	1.81 ± 0.03	1.08 ± 0.01	1.34 ± 0.06	1.48 ± 0.07	2.08 ± 0.07	2.31 ± 0.07
Concentration of residual glucose (g L^{-1})	15.0 ± 0.43	22.9 ± 0.13	16.6 ± 0.12	12.1 ± 0.14	17.6 ± 0.20	12.0 ± 0.10
$Y_{P/S}$ (g g^{-1})	0.116	0.137	0.104	0.073	0.149	0.129
$Y_{X/S}$ (g g^{-1})	0.61	0.55	0.44	0.55	0.47	0.98
EPS productivity (g L^{-1} h^{-1})	0.0151	0.0064	0.0093	0.0088	0.0173	0.0240

was similar to that previously reported by Wu *et al.*[15], who indicated that a lower initial pH (pH 5.0) was beneficial to biomass accumulation, and that a higher initial pH (pH 5.5) contributed to EPS production by *A. auricula*.

EPS production with two-stage pH control strategy

The pH level was also considered as a trigger factor for large-scale fermentation system[25]. To achieve maximum metabolite formation, two-stage pH control strategy had been carried out in the fermentation for the biosynthesis of various products such as polysaccharide[14,15,19–21], 2,3-butanediol[24], amino butyric acid[25], e-poly-L-lysine[26], arachidonic acid[27], cutinase[28], etc.

The time course of the proposed pH-shift strategy for EPS production by *H. marmoreus* is shown in Fig. 5. The kinetic parameters of different pH strategy by *H. marmoreus* in a 5-L bioreactor are summarized in Table 2. As shown in Table 2, the maximal biomass (12.3 g L^{-1}) was achieved after 120 h under the two-stage pH control fermentation, which was respectively 44.7 %, 16.0 % and 50.0 % higher than that of fermentation under natural pH, constant pH control at pH 6.5 and pH 7.5. Furthermore, the two-stage pH control strategy not only considerably improved EPS production but also increased EPS productivity compared with natural pH and constant pH control (pH 6.5 and pH 7.5) cases. The maximal EPS concentration and productivity reached 2.31 ± 0.07 g L^{-1} and 0.024 g L^{-1} h^{-1}, which were 27.62 % and 58.94 % higher than that obtained at natural pH fermentation, respectively. In addition, the residual glucose concentration (12.0 ± 0.10 g L^{-1}) in the pH-shift control strategy was much lower than that of natural pH culture and con-

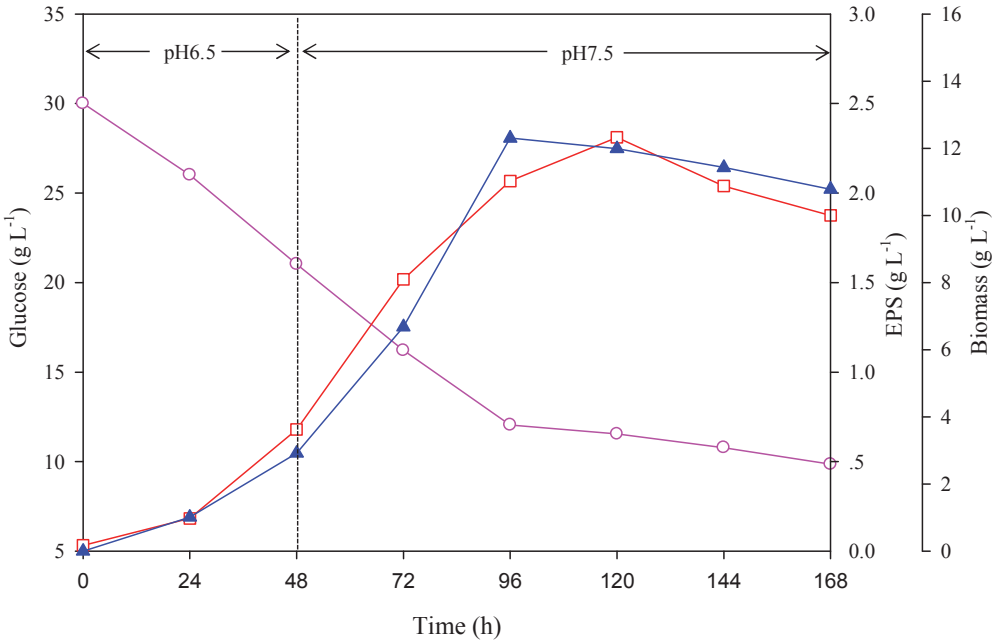

Fig. 5 – *Time courses of submerged culture of H. marmoreus in 5-L fermenter under two-stage pH strategy. EPS (▲), biomass (□) and glucose (○).*

stant pH control fermentation at pH 7.5. These results showed that the proposed pH-shift strategy could efficiently improve the mycelial growth, glucose consumption, and EPS production in submerged culture of *H. marmoreus*.

Conclusions

The pH 6.5 was suitable for the mycelial growth in submerged culture of *H. marmoreus* WZ019, but pH 7.5 was beneficial to EPS production under the optimal media containing (g L^{-1}): glucose 30, yeast extract 12.5, K_2HPO_4 1.5, $MgSO_4$ 0.75. A two-stage controlled pH strategy for EPS production has been developed based on the kinetic parameters analysis. This method (pH switching from 6.5 to 7.5) was proven to be superior to the constant pH and the natural pH cultures in terms of maximal EPS concentration and productivity.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

This article contains no studies with human or animal subjects.

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