Fed-batch fermentation of *Tuber melanosporum* for the hyperproduction of mycelia and bioactive *Tuber* polysaccharides

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**A R T I C L E   I N F O**

Article history:
Received 17 December 2008
Received in revised form 17 February 2009
Accepted 18 February 2009
Available online 20 March 2009

Keywords:
Medicinal mushroom
*Tuber melanosporum*
Fed-batch fermentation
*Tuber* polysaccharides

**A B S T R A C T**

For the first time, a fed-batch fermentation process of *Tuber melanosporum* was developed for the efficient production of bioactive mycelia and *Tuber* polysaccharides. Each 1.67 g/L of peptone and 8.33 g/L of yeast extract were added on day 3, 6, and 9, respectively, and sucrose was fed to maintain its concentration around 35–5 g/L when its residual level decreased to 10–5 g/L. Then, the maximal biomass, the production of extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS) reached 53.72 ± 2.57 g DW/L, 7.09 ± 0.62 and 4.43 ± 0.21 g/L, respectively. Compared with the batch culture conducted in the enriched medium, the biomass, the production of EPS and IPS were enhanced by 55.8%, 222.3% and 103.2%, respectively. Not only the cell density but also the production of EPS and IPS were the highest ever reported in truffle fermentation, and the biomass was also the highest as ever reported in mushroom fermentation.

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1. Introduction

*Tuber melanosporum*, also known as “black diamonds”, which lives in symbiosis with plant host roots in order to accomplish its life cycle, belonging to the family Tubercular, the order Tuberales, and the phylum Ascomycotina (Tang et al., 2007a; Hawksworth et al., 1995). *T. melanosporum* is highly appreciated in many countries because of its special taste and smell (Mello et al., 2006). Hu et al. (1994) showed that *Tuber* polysaccharides isolated from the fruiting-body have immunomodulating and anti-tumor activities (Hu et al., 1994). Because it usually takes 7–9 years to cultivate the fruiting-body of *T. melanosporum*, which is a time-consuming and quality-fluctuating process (Hall et al., 2003), submerged culture is viewed as a promising alternative for the efficient production of bioactive metabolites (Tang et al., 2007a,b).

Truffle fermentation to produce mycelia, extracellular polysaccharides (EPS), and intracellular polysaccharides (IPS) was developed for the first time in our lab (Tang et al., 2008a,b; Liu et al., 2008). Liu et al. (2008) demonstrated that the maximal cell density of 23.94 ± 0.49 g/L was achieved by the combination of 10 g/L of peptone and 30 g/L of yeast extract. Tang et al. (2008a) indicated that the highest EPS production of 5.86 ± 0.21 g/L was obtained when 30 mM Mg2+, 5 mM K+ metal ion, and 80 g/L of sucrose were used. Besides, the highest IPS production of 2.92 ± 0.02 g/L was obtained at an initial sucrose concentration of 80 g/L (Tang et al., 2008b).

Based on our previous results, an enriched medium (i.e., 80 g/L sucrose, 10 g/L peptone, 30 g/L yeast extract, 5 mM K+, and 30 mM Mg2+) was proposed in order to further enhance the cell density and the production of EPS and IPS. The cell growth was improved by the enriched medium, while the production of *Tuber* polysaccharides was dramatically decreased. Hence, a fed-batch fermentation process was proposed to figure out the problem in this work. By using three different feeding strategies, (i.e., nitrogen source pulse feeding strategy, carbon source pulse feeding strategy, nitrogen and carbon source mixing feeding strategy), the cell density, the production of EPS and IPS were significantly improved, and the biomass and *Tuber* polysaccharides production were the highest in truffle fermentation. The information obtained in this work is useful for the large-scale fermentation of *T. melanosporum*.
2. Methods

2.1. Maintenance and preculture of T. melanosporum

The strain of T. melanosporum was kindly provided by Mianyang Institute of Edible Fungi (Sichuan Province, China). It was maintained on potato-agar-dextrose slants. The slant and preparation of the preculture have been previously described (Tang et al., 2008a; Liu et al., 2008).

2.2. Batch fermentation in the enriched medium

Based on our previous work, the proposed enriched medium consisted of the following components: sucrose, 80 g/L; peptone, 10 g/L; yeast extract, 30 g/L; Vitamin B1, 0.05 g/L; K⁺, 5 mM; and Mg²⁺, 30 mM. Compared with the enriched medium, the initial concentration of yeast extract was decreased from 30 g/L to 5 g/L, and the initial concentration of peptone was decreased from 10 g/L to 5 g/L. So, 5 g/L of peptone and 25 g/L of yeast extract was fed during the fermentation process. In order to optimize nitrogen source pulse feeding, three strategies were tested. Firstly, 5 g/L of peptone and 25 g/L of yeast extract was fed into the fermentation system after the culture of day 3 (hereafter referred to as one-time nitrogen source feeding). Secondly, each 2.5 g/L of peptone and 12.5 g/L of yeast extract were added into the fermentation system after the culture of day 3 and day 6, respectively (hereafter referred to as two-time nitrogen source feeding). Finally, each 1.67 g/L of peptone and 8.33 g/L of yeast extract were added into the fermentation system after the culture of day 3, day 6, and day 9, respectively (hereafter referred to as three-time nitrogen source feeding). Loading volume in shake flask was kept constant at 50 mL after feeding. The control experiment was conducted without nitrogen source feeding for comparison. The other culture conditions were the same as our previous report (Tang et al., 2008a).

2.3. Nitrogen source pulse feeding experiments

The initial fermentation medium consisted of the following components: sucrose, 35 g/L; peptone, 5 g/L; yeast extract, 5 g/L; Vitamin B1, 0.05 g/L; K⁺, 5 mM; and Mg²⁺, 30 mM. Compared with the enriched medium, the initial concentration of sucrose was decreased from 80 g/L to 35 g/L. When the residual sugar concentration was decreased to 10–5 g/L during the fermentation, a certain concentration of sucrose solution was fed to maintain the residual sucrose concentration around 35–5 g/L. Loading volume in shake flask was kept constant at 50 mL after feeding. The control experiment was conducted without carbon source feeding for comparison. The other culture conditions were the same as our previous report (Tang et al., 2008a).

2.4. Carbon source pulse feeding experiments

The initial fermentation medium consisted of the following components: sucrose, 35 g/L; peptone, 10 g/L; yeast extract, 30 g/L; Vitamin B1, 0.05 g/L; K⁺, 5 mM; and Mg²⁺, 30 mM. Compared with the enriched medium, the initial concentration of sucrose was decreased from 80 g/L to 35 g/L. When the residual sugar concentration was decreased to 10–5 g/L during the fermentation, a certain concentration of sucrose solution was fed to maintain the residual sucrose concentration around 35–5 g/L. Loading volume in shake flask was kept constant at 50 mL after feeding. The control experiment was conducted without carbon source feeding for comparison. The other culture conditions were the same as our previous report (Tang et al., 2008a).

2.5. Carbon and nitrogen sources mixing feeding experiment

Carbon and nitrogen sources mixing feeding experiment was conducted by combining the optimal nitrogen and carbon source feeding strategies (hereafter referred to as mixing feeding). The initial fermentation medium consisted of the following components: sucrose, 35 g/L; peptone, 5 g/L; yeast extract, 5 g/L; Vitamin B1, 0.05 g/L; K⁺, 5 mM; and Mg²⁺, 30 mM. The control experiment was conducted without feeding for comparison. The other culture conditions were the same as our previous report (Tang et al., 2008a).

2.6. Sampling, determination of dry cell weight and residual sugar concentration

For sampling, three flasks were taken each time. The methods to determine dry cell weight (DCW) and residual sugar concentration were the same as previous works (Tang et al., 2008a; Liu et al., 2008).

2.7. Measurements of extracellular and intracellular polysaccharides

For the determination of extracellular polysaccharides (EPS) and intracellular polysaccharides (IPS), the methods were the same as previous works (Tang et al., 2008a; Liu et al., 2008).

3. Results and discussion

3.1. Batch fermentation in the enriched medium

During the batch fermentation conducted in the enriched medium (i.e., 80 g/L sucrose; 10 g/L peptone; 30 g/L yeast extract; 0.05 g/L Vitamin B1; 5 mM K⁺; and 30 mM Mg²⁺), the maximal biomass was 34.49 ± 0.33 g DW/L, which was enhanced by 44.1% compared with the highest cell density of 23.94 ± 0.49 g DW/L obtained in the medium (i.e., 60 g/L glucose, 10 g/L peptone, 30 g/L yeast extract, 0.05 g/L Vitamin B1, 7.4 mM K⁺, and 2 mM Mg²⁺) (Liu et al., 2008). Tang et al. (2008a) demonstrated that the concentration of K⁺ or Mg²⁺ did not significantly affect the cell growth of truffle. This indicated that higher initial sucrose concentration was optimal for T. melanosporum growth.

The maximal EPS production obtained in the enriched medium was 2.20 ± 0.06 g/L, which were decreased by 62.5% compared with the highest value of 5.86 ± 0.21 g/L obtained in the medium (i.e., 80 g/L sucrose, 5 g/L peptone, 5 g/L yeast extract, 0.05 g/L Vitamin B1, 5 mM K⁺, and 30 mM Mg²⁺) (Tang et al., 2008a), and, the maximal IPS production obtained in the enriched medium was 2.18 ± 0.14 g/L, which were decreased by 25.3%, compared with the highest value of 2.92 ± 0.20 g/L obtained in the medium (i.e., 80 g/L sucrose, 5 g/L peptone and 5 g/L yeast extract, 0.05 g/L Vitamin B1, 7.4 mM K⁺, 2 mM Mg²⁺) (Tang et al., 2008b). This demonstrated that Tuber polysaccharides (i.e., EPS and IPS) biosynthesis were seriously inhibited by relatively higher initial nitrogen source concentration (i.e., 10 g/L peptone, 30 g/L yeast extract).

3.2. Effect of nitrogen source pulse feeding strategy

In order to avoid the above inhibitory effects, nitrogen source feeding was adopted during fermentation. As shown in Fig. 1A, T. melanosporum growth was significantly improved by nitrogen source feeding. The maximal cell concentration of 37.35 ± 0.07, 39.50 ± 0.93, and 41.65 ± 2.44 g DW/L was obtained after the culture of day 12, 14, and 12 by one-time, two-time and three-time nitrogen source feeding, respectively, which was enhanced by 71.0%, 80.9% and 90.7% compared with the batch fermentation.

As depicted in Fig. 1B, culture pH variation pattern was significantly affected by nitrogen source feeding. For one-time nitrogen source feeding, culture pH increased from 3.0 to around 5.6 after feeding on day 3, and maintained the following 8 days, and then increased quickly to around 8.0 at the end of fermentation. Interestingly, for three-time nitrogen source feeding, culture pH has a
linearity increase since its first feeding on day 3. These results suggested the feeding of organic nitrogen source caused culture pH increase. However, ammonium feeding in the culture of *Cordyceps sinensis* caused a sharp drop of medium pH (Leung and Wu, 2007).

Fig. 2A shows EPS accumulation pattern under various nitrogen source pulse feeding strategies were quite similar. The maximal EPS production titer of 1.73 ± 0.04, 1.39 ± 0.04, and 1.46 ± 0.07 g/L was obtained by one-time, two-time, and three-time nitrogen source feeding, respectively. While, these were all much lower than that of 3.50 ± 0.15 g/L obtained in the batch fermentation. The results indicated that nitrogen source feeding was not beneficial for EPS biosynthesis. However, Leung and Wu (2007) reported EPS production was increased from 2.6 to 3.7 g/L by NH$_4$ feeding in the fermentation of *C. sinensis*. Besides, a stimulatory effect of NH$_4$ on cordycepin biosynthesis in submerged fermentation of *C. militaris* was also reported by Mao and Zhong (2006). As shown in Fig. 2B, nitrogen source pulse feeding strategy did not significantly affect the total IPS accumulation.

To conclude, a three-time nitrogen source pulse feeding strategy was optimal for *T. melanosporum* growth, and its maximal cell density of 41.65 ± 2.44 g DW/L was 90.7% higher compared with the batch fermentation. While, the biosynthesis of *Tuber* polysaccharides was significantly inhibited by nitrogen source feeding.

3.3. Effect of carbon source feeding

As described above, it seems that a relatively higher sucrose concentration (i.e., 80 g/L) was not suitable for *Tuber* polysaccharides accumulation even by using various nitrogen source pulse feeding strategy. Then, sucrose pulse feeding was tested in order to improve *Tuber* polysaccharides biosynthesis. As depicted in Fig. 3A, a total amount of 185.6 g/L sucrose was fed to *T. melanosporum* fermentation process by six pulse feedings. Fig. 3B shows the cell growth of *T. melanosporum* was significantly improved by sucrose feeding. The maximal cell concentration reached 50.15 ± 1.10 g DW/L, which was enhanced by 111.4% compared with the batch fermentation. After the culture of 25 days, the biomass began to decrease, so sucrose feeding stopped. Fig. 3B indicates that culture pH in the fed-batch fermentation was much lower than the batch fermentation.

As depicted in Fig. 4, the maximal EPS production of 3.40 ± 0.12 g/L was obtained by sucrose feeding, which was higher by 98.8% compared with the batch fermentation (i.e., 1.71 ± 0.19 g/L). This clearly demonstrated that sucrose feeding was favorable to EPS biosynthesis. In the shake flask culture of *Ganoderma lucidum*, Tang and Zhong (2002) also reported EPS production was improved to 0.75 ± 0.05 g/L by lactose feeding, which was 41.5% higher than the batch culture. The similar positive effects of carbon source feeding on EPS production were also observed in the fermentation of *Ganoderma resinaceum* DG-6556 (Kim et al., 2006), *Agaricus bra-*
Siliensis (Zou, 2006), Grifola frondosa (Shih et al., 2008), and Tremella mesenterica NRRL Y-6785 (Baets et al., 2002).

Fig. 4 also demonstrates that the maximal IPS production achieved 4.05 ± 0.21 g/L by sucrose feeding, which was higher by 108.8% compared with the batch culture (i.e., 1.94 ± 0.38 g/L). The result indicated that sucrose feeding was favorable to enhance IPS production due to a relatively high cell density obtained in the fed-batch fermentation. The similar result was also observed in the shake flask culture of G. lucidum, Tang and Zhong (2002) reported IPS production was improved to 2.40 ± 0.05 g/L by lactose feeding, which was 40.4% higher than the batch culture.

To conclude, by sucrose feeding, the highest biomass (i.e., 50.15 ± 1.10 g DW/L), EPS production (i.e., 3.40 ± 0.12 g/L) and IPS production (i.e., 4.05 ± 0.21 g/L) were obtained, which were increased by 111.4%, 98.8% and 108.8% compared with the batch culture. These demonstrated that sucrose feeding was very useful to enhance the cell density and Tuber polysaccharides accumulation.

3.4. Effect of nitrogen and carbon sources mixing feeding

As we know, both carbon and nitrogen sources and their balance are very important for metabolite biosynthesis. In the culture

![Fig. 3. Effect of carbon source pulse feeding on sucrose consumption (A), T. melanosporum growth and culture pH variation (B). Symbols: (A) the fed-batch fermentation by sucrose feeding (dark square, ■), the batch fermentation for control (open square, □); (B) the cell growth by sucrose feeding (dark circle, ○) and the control (open circle, □), the culture pH by sucrose feeding (dark triangle, ▲) and the control (open triangle, △). The error bars in the figure show the standard deviation from three independent samples.](image1)

![Fig. 4. Effect of carbon source pulse feeding on the production of EPS and IPS. Symbols: the EPS production by sucrose feeding (dark triangle, ▲) and the control (open triangle, △), the IPS production by sucrose feeding (dark circle, ●) and the control (open circle, ○).](image2)

![Fig. 5. Kinetic profiles of sucrose consumption (A), T. melanosporum growth and culture pH (B) under nitrogen and carbon sources mixing feeding strategy. Symbols: (A) the fed-batch fermentation by mixing feeding (dark square, ■), the batch fermentation for control (open square, □); (B) the cell growth by mixing feeding (dark circle, ●) and the control (open circle, ○), the culture pH by mixing feeding (dark triangle, ▲) and the control (open triangle, △). The error bars in the figure show the standard deviation from three independent samples.](image3)
of *T. sinense*, Liu et al. (2008) observed a significant synergic effect of carbon and nitrogen sources on the cell growth and *Tuber* polysaccharides biosynthesis. So, the performance of a three-time nitrogen source pulse feeding combined with sugar pulse feeding was investigated.

Time profile of residual sucrose concentration is shown in Fig. 5A. A total amount of 279.6 g/L sucrose was fed to the fermentation process of *T. melanosporum* by nine pulse feedings. Fig. 5B clearly shows that the cell growth of *T. melanosporum* was significantly improved by carbon and nitrogen sources mixing feeding. The maximal cell density of 53.72 ± 2.57 DW/L was obtained after the culture of day 36, which was higher by 230.4% compared with the batch culture (i.e., 16.26 ± 0.84 g/L). After the culture of 38 days, a gradual decrease of biomass was observed, so sucrose feeding stopped (Fig. 5A). As depicted in Fig. 5B, culture pH in the mixing feeding experiment was similar with the three-time nitrogen pulse feeding experiment. The results demonstrated the feeding of organic nitrogen source caused a rapid and sharp increase in culture pH.

Fig. 6 indicates that EPS production was significantly enhanced by mixing feeding. The maximal EPS production reached 7.09 ± 0.62 g/L, which was higher by 213.7% compared with the batch fermentation (i.e., 2.26 ± 0.09 g/L). Fig. 6 demonstrates that the total IPS accumulation was also significantly enhanced by mixing feeding. The maximal IPS production was 4.43 ± 0.21 g/L, which was higher by 246.1% compared with the batch fermentation (i.e., 1.28 ± 0.20 g/L).

In conclusion, by carbon and nitrogen sources mixing feeding strategy, the maximal cell density of 53.72 ± 2.57 g DW/L, EPS production of 7.09 ± 0.62 g/L and IPS production of 4.43 ± 0.21 g/L were obtained, which were enhanced by 230.4%, 246.1% and 246.1%, respectively, compared with the batch culture. Compared with the three feeding strategies, the highest biomass, the production of EPS and IPS were obtained by using the carbon and nitrogen sources mixing feeding strategy, which was enhanced by 29.0%, 385.6% and 57.7% compared to the three-time nitrogen source feeding, respectively, which also was 7.1%, 108.5% and 9.4% higher than those obtained by using carbon source feeding, respectively. These demonstrated that mixing feeding strategy was very efficient to enhance the cell density and *Tuber* polysaccharides accumulation.

**4. Conclusions**

In this work, a fed-batch fermentation process of medicinal mushroom *T. melanosporum* for the efficient production of bioactive mycelia and *Tuber* polysaccharides was developed. Inhibition of *Tuber* polysaccharides biosynthesis by a relatively higher initial concentration of sucrose (i.e., 80 g/L), peptone (i.e., 10 g/L) and yeast extract (i.e., 30 g/L) were avoided. Not only the cell growth, but also the production of EPS and IPS were markedly improved by carbon and nitrogen sources mixing feeding strategy, and the maximal cell density (i.e., 53.72 ± 2.57 g DW/L), EPS production (i.e., 7.09 ± 0.62 g/L) and IPS production (i.e., 4.43 ± 0.21 g/L) were obtained, which was enhanced by 55.8%, 222.3% and 103.2%, respectively, compared with the batch fermentation in the enriched medium. The fundamental information obtained in this work is useful for the production of *Tuber* polysaccharides and mycelia biomass in large-scale. Such work may also be helpful to other mushroom fermentations for useful metabolite production.

**Acknowledgements**

The financial supports from the National Natural Science Foundation of China (NSFC, Project No. 20706012), National High Technology Research and Development Key Program of China (Project No. 2007AA021506), the Scientific Research Foundation for the Returned Overseas Chinese Scholars (Ministry of Personnel), Hubei Provincial Innovative Research Team in University (Project No. T200608), Hubei Provincial Natural Science Foundation for Innovative Research Team (Project No. 2006CDA002), Hubei Provincial International Cooperation Foundation for Scientific Research (Project No.2007CA012), the Scientific Research Key Foundation from Hubei University of Technology (Project No. 306.18002), and the Open Project Program of the State Key Laboratory of Bioreactor Engineering (ECUST) are gratefully acknowledged. Ya-Jie Tang also thanks the Chutian Scholar Program from Hubei Provincial Department of Education, China (2006).

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