Two-step Shake-static Fermentation to Enhance Cordycepin Production by *Cordyceps militaris*

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Production of cordycepin by *Cordyceps militaris* batch fermentation was investigated under various fermentation styles. Two-step shake-static fermentation strategy was proposed to achieve the optimal cordycepin production. It can be assumed that cordycepin production is linked to the development of aerial hyphae and conidiophores by analyzing the results of kinetics and morphology of *C. militaris* fermentation. Massive aerial hyphae and conidiophores accumulation requires two conditions: adequate *C. militaris* biomass as an essential material foundation and the environmental condition, static fermentation in this case, which can induce cellular differentiation to form aerial hyphae and conidiophores. The specific mycelia growth rate was higher with sufficient nutrition and dissolved oxygen in shake fermentation, and the specific cordycepin production rate was higher in static fermentation. Under two-step shake-static fermentation, a lot of *C. militaris* cells were obtained in shake stage and the development of conidiophore was activated in static stage, followed by the accumulation of cordycepin. Therefore, cordycepin production reached 2.62 g/L.

1. Introduction

Cordycepin, an adenosine analogue, is a nucleic acid antibiotic extracted from *Cordyceps militaris (Yue et al., 2013)*. It has the anti-tumor, anti-leukemic, anti-metastatic, anti-bacterial, anti-viral, anti-trypanosomiasis, anti-restenosis, immunomodulatory, and anti-inflammatory activities (Paterson, 2008). Cordycepin is synthesized commercially via solid state fermentation and submerged *C. militaris* cultivation (Das et al., 2010b). Considerable effort is currently focused on three aspects of cordycepin production: strain screening and improvement (Das et al., 2008), additives (Fan et al., 2012; Mao & Zhong, 2006; Masuda et al., 2007) and optimizing fermentation process (Das et al., 2010a; Mao et al., 2005; Mao & Zhong, 2004; Masuda et al., 2011; Masuda et al., 2007; Masuda et al., 2006; Shih et al., 2007).

In general, the secondary metabolites synthesis can be considered a non-growth-associated process. Because secondary metabolites are not directly required to ensure the growth of the organisms that produce them, these products accumulation are not relevant to cell growth. However, the simple non-growth-associated process is not applicable in higher fungi, especially *C. militaris*. *C. militaris* mating and sexual cycles are often complicated, as well as its asexual reproduction and morphological differentiation (Zheng et al., 2011). Additionally, the different distribution of cordycepin in fruit bodies demonstrates that the capacity of *C. militaris* to produce cordycepin can be different in various cells types (Dong et al., 2013). At this point, cordycepin production of *C. militaris* was similar with ganoderic acids production of *Ganoderma lucidum*. Therefore, cordycepin production was studied based on the combination of fermentation kinetics and morphological change of *C. militaris*.

In addition, there is the relationship between secondary metabolism and fungal development. Some secondary metabolites stimulate sporulation and therefore influence the development of the producing organism. Natural products are often produced late in fungal development, and their biosynthesis is complex. A common signal transduction pathway can exist to be partially responsible for tying fungal development to natural product biosynthesis (Calvo et al., 2002). Therefore, the relationship between cordycepin production and fungal development should be investigated in *C. militaris* fermentation.

In this study, the course and cell morphology of various fermentation styles were compared and analyzed to achieve a more efficient process for cordycepin production. The specific growth rate and production rate were

calculated on *C. militaris* fermentation in order to obtain useful information for large-scale production of secondary metabolites by the bioprocess.

2. Materials and methods

2.1 Microorganism and media
C. militaris 14014 purchased from the China Center of Industrial Culture Collection was stored at 4 °C. The strain was inoculated on potato–agar–dextrose (PDA) slants or plate containing 20.0 g/L glucose, 3.0 g/L KH_2PO_4, 1.5 g/L $MgSO_4·7H_2O$, and a small amount of Vitamin B1. The slants and plates were then incubated at 25 °C for 7 d. The liquid medium was composed of 40 g/L glucose, 10 g/L tryptone, 6 g/L yeast extract, 0.5 g/L $MgSO_4·7H_2O$, 0.5 g/L $K_2HPO_4·3H_2O$, and 0.5 g/L KH_2PO_4. Approximately 50 mL of the medium was placed in a 250 mL flask and sterilized.

2.2 Spore suspension preparation
50 mL of fresh liquid medium was added into an active PDA slant from the stock culture. The spores were scraped using a sterilized inoculating loop, and then the medium containing spores was introduced into a sterile and dry 250 mL Erlenmeyer flask. The culture was incubated at 25 °C on a rotary shaker at 180 rpm for 4 d. Liquid culture was filtered through four layers of sterile gauze into a sterile tube and centrifuged to remove the supernatant. The precipitate was diluted to $1.0×10^5$ cfu/mL of spore suspension with sterile water. The spore concentrations were quantified with a hemacytometer.

2.3 Inoculation and culture
Spore suspensions as inocula were added into the fresh basal medium in the shake (180 rpm) and static cultures. Fermentation temperature was 27 °C. All experiments were conducted in triplicate at least.

2.4 Analytical methods
The entire contents of the culture flask were centrifuged. The collected mycelia were then sufficiently washed with distilled water and dried at 110 °C in an oven until a constant dry weight was obtained. After centrifuging, one part of the supernatant was stored at -20 °C and later thawed for analyses of residual sugar and cordycepin. Glucose content of the culture was determined using 3,5-dinitryl-salicylic acid reagent. Cordycepin concentrations were measured using a high-performance liquid chromatograph (LC-20AD system, Shimadzu Corp., Japan) on a reverse phase column (5 µm Ultimate AQ-C18 column, 250 mm × 4.6 mm) (Tang et al., 2014).

2.5 Morphology observation
The morphology of mycelia was observed by SEM (JSM-6510, JEOL, Japan). The cultured mycelia in the fermentation broth were collected using centrifugation and wased with sterile water, followed by lyophilization. Samples were sliced into thin sections and then gold sputtered before SEM imaging.

3. Results and discussion

3.1 Effect of fermentation styles on cells growth, glucose consumption, pH and cordycepin production
As shown in Figure 1, the effects of fermentation styles on cells growth, glucose consumption, pH and cordycepin production are indifferent. The biomass dry weight increased rapidly at the beginning of shake fermentation (SHF). The average cell proliferation rate of *C. militaris* 14014 reached 6.79 g/L/d during the first three days of fermentation and maximum mycelial dry weight was about 23.78 g/L on day 5. Specific cell growth rate reaches 4.42-4.77 d^{-1} in the initial stage of shake condition (Figure 2). However, *C. militaris* mycelia on the medium surface grew very tardily in static fermentaion (STF), when exposed to the atmosphere. Specific cell growth rate is about 0.75 d^{-1}, which is much lower than that of SHF. Mycelial dry weight was approximately 10.00 g/L on day 29.

In addition, carbon source consumed much more quickly in SHF than STF. The result suggested that most glucose was utilized for cell growth in SHF. Carbon source was nearly depleted before day 5 of SHF. During the SHF that followed, intermediate products were completely oxidized to carbon dioxide and water through aerobic respiration and were not transformed into cordycepin. Figure 2 shows that specific cordycepin production rate reduces to zero after 7 d, indicating that cells stopped to synthetise cordycepin. Therefore, the cordycepin production rate was lower in SHF than STF after day 5 of fermentation. The medium pH became lower in STF than SHF, which meant that anaerobic respiration dominated in carborn metabolism and cells produced more acids (Tabak & Cooke, 1968). Carb on source was gradually used for cordycepin production in the later period of STF. The maximum cordycepin production of 0.51 g/L and 1.03 g/L were achieved in SHF and STF, respectively.
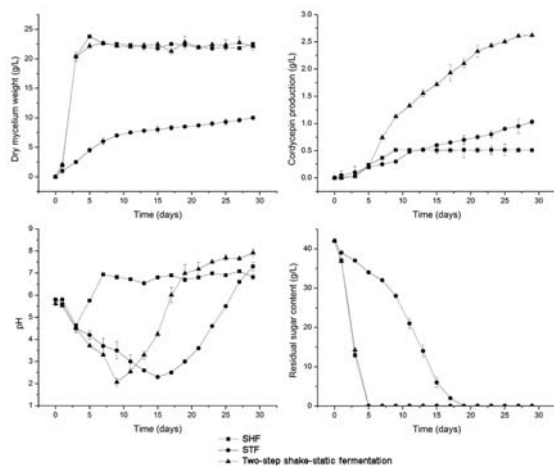
Figure 1: Time profiles of mycelial growth, cordycepin production, pH and glucose consumption during batch fermentation of C. militaris 14014 at various fermentation styles in a flask. The inoculum size of C. militaris 14014 spore suspension was 10% v/v.

On the basis of the above analysis, a two-step shake-static fermentation strategy was developed. SHF carried out for the first 4 days of fermentation, and then it was shifted to STF. When fermentation style transformed to STF after shaking for 4 d, cells rapidly swallowed up the dissolved oxygen in fermentation broth so that intracellular aerobic respiration was converted to anaerobic respiration. Even though residual sugar was zero, intermediates inside cells were transferred to the pathway of anaerobic respiration and then converted into organic acids (Tabak & Cooke, 1968), resulting in the pH decrease of fermentation broth, which accumulated some precursors for cordycepin synthesis. Whether the early stage of fermentation was shake culture or static culture, specific cordycepin synthetic rate maintained in the range of 0.002-0.005 d^{-1} through a saddle-shaped curve in the later period of fermentation (Figure 2). Moreover, the decrease of dissolved oxygen in fermentation broth could induce the cells differentiation (Scott & Eaton, 2008). When spores developed to substrate mycelia after day 7 of STF, the mass transfer resistance of nutrition and dissolved oxygen would occur and lead to the transformation from substrate mycelia to aerial hyphae and advanced cordycepin accumulation. But the hypoplasia of substrate mycelia later led to the failure to transport the nutrition to aerial hyphae and conidiophores, so the specific production rate decreased again to form a saddle-shaped curve. Similarly, lower dissolved oxygen induced substrate mycelia to extend away from air-liquid interface in day 4 of two-step shake-static fermentation until aerial mycelium and conidiophore was developed. Subsequently, the mass transfer was limit ed to lead to a saddle-shaped curve. Then, conidiophores generated conidia through microcycle conidiation (Kepler et al., 2012; Zheng et al., 2011). The combination of aerial hyphae, conidiophores and conidia formed colonies and mycelia pad (Figure 3), which was essentially similar to colonial morphology in repeated batch culture (Das et al., 2010b; Masuda et al., 2014).
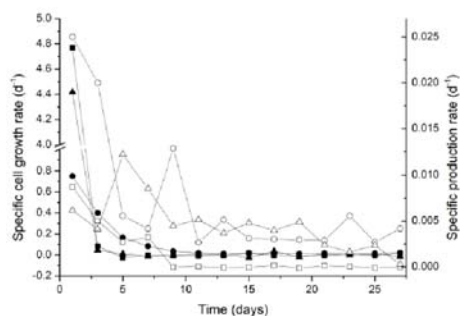

Figure 2: Specific cell growth rate and cordycepin production rate of C. militaris with various fermentation styles. Solid represents the specific cell growth rate and hollow represents the specific production rate. Square, circular and triangle represent shake, static and two-step shake-static fermentation, respectively

Figure 3: Colonies and mycelia pad of C. militaris in two-step shake-static fermentation.

3.2 Morphology observation

Due to the difference of cordycepin production in various cell types, the mycelial micromorphology of *C. militaris* was observed by SEM. Figure 4A-C show that the mycelia are thick and smooth, having many articulate branches under SHF. Their diameters are in the range of 2-5 μm and the gaps among the mycelia are small. They have the typical characteristics of substrate mycelium. However, Figure 4D-F indicates that the mycelia are long and thin, owning little branches under STF. Their diameters are about 1-2 μm and the gaps are big. They have the rough surface with many wrinkles. The mycelia possess the typical characteristics of aerial hyphae and conidiophores.

Secondary metabolism is associated with fungal developmental processes (Calvo et al., 2002). According to the data of fermentation kinetics and mycelial morphological differentiation, the development of aerial hyphae and conidiophores is linked to high cordycepin production. Once cells differentiated and entered aerial mycelium and conidiophore stage, the cordycepin production rate reached a plateau. The aerial mycelium and conidiophore developed from the substrate mycelia of the biomass, so the amount of aerial mycelia and conidiophores essentially depended on that of biomass. The results showed that a lot of biomass accumulated in SHF, but then no trace of aerial hyphae and conidiophores was found because of lacking the condition, while low cordycepin production occurred; When inoculated with spore suspension, the total quantity of spores was too little. Despite aerial hyphae and conidiophores were well-developed in STF, a small quantity of developed aerial hyphae and conidiophores seriously affected the cordycepin accumulation; During two-step shake-static fermentation, a large number of biomass accumulated in the shake stage to lay a good foundation of aerial hyphae and conidiophores development in static stage. Aerial hyphae and conidiophores developed well under the condition of STF and cordycepin production reached 2.62 g/L.

C. militaris produce cordycepin efficiently at a time that coincides with the mycelia development and differentiation. In C. militaris, these processes can be regulated by a common signaling pathway, like other fungi (Calvo et al., 2002). In the previous work, two-stage dissolved oxygen control strategy was developed for hyperproduction of cordycepin in submerged cultivation (Mao & Zhong, 2004). The strategy and two-step shake-static fermentation strategy can both be based on the hypothetic signaling pathway. The only difference is that the cells are hungry due to the mass transfer resistance under STF in this study. The cell starvation may help to improve the mycelia development and cordycepin production.
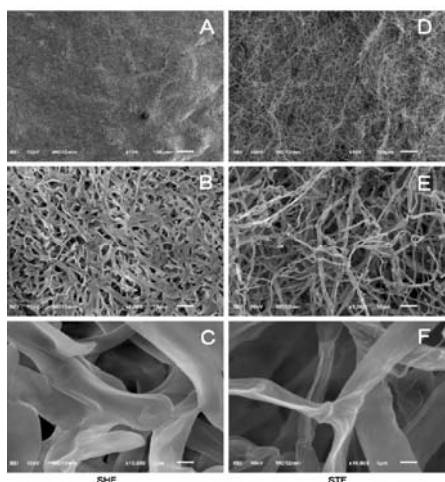

Figure 4: Mycelial morphology of C. militaris on SHF and STF.

4. Conclusions

The results of fermentation kinetics of Cordyceps militaris showed the difference of cell growth, development and cordycepin biosynthesis in SHF and STF. The mycelia grew much faster in SHF. However, STF benefitted cells development and cordycepin production. According to SEM observation, it was found that the substrate mycelia of *C. militaris* in STF more easily differentiated aerial hyphae and conidiophores, accompanied by the synthesis of a large number of cordycepin. The present study clarified the key factors in high cordycepin production and the relationship between cellular morphology and cordycepin accumulation. High cordycepin yield was linked to the mycelia development at the genetic level. The optimal cordycepin production was achieved in two-step shake-static fermentation owing to aerial hyphae and conidiophores development.

Acknowledgements

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