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Optimization of carbon source and carbon/nitrogen ratio for cordycepin production by submerged cultivation of medicinal mushroom *Cordyceps militaris*

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Abstract

Effects of various carbon sources and carbon/nitrogen ratios on production of a useful bioactive metabolite, cordycepin (3′-deoxyadenosine), by submerged cultivation of a Chinese traditional medicinal mushroom *Cordyceps militaris* were investigated in shake flasks. The carbon sources examined were lactose, sucrose, glucose, fructose, galactose, maltose and xylose, and glucose was found to be most favourable to cordycepin production, whereas cells grew best in galactose medium. The dry cell weight (DW) was increased with an increase in initial glucose concentration within the range of 25–70 g/l as investigated. The highest cordycepin production, i.e. 245.7 ± 4.4 mg/l on day 18, was obtained in medium containing 40 g glucose/l. To enhance further the cordycepin production, the effect of carbon/nitrogen ratios was studied using central composite design and response surface analysis. The maximum cordycepin production and productivity of 345.4 ± 8.5 mg/l and 19.2 ± 0.5 mg/l per day were achieved in medium with optimized carbon and nitrogen sources, i.e. 42.0 g glucose/l and 15.8 g peptone/l. The information obtained is helpful for the hyperproduction of cordycepin by submerged cultivation of *C. militaris* on a large scale.

Keywords: Medicinal mushroom; Submerged cultivation; Cordycepin; *Cordyceps militaris*; Response surface analysis; Central composite design

1. Introduction

Mushroom is an abundant source of a wide range of useful natural products with biological activities. Since the field cultivation of mushroom takes several months to yield the fruiting body with a low productivity of bioactive compounds, submerged cultivation of mushrooms is viewed as a promising alterative for producing valuable substances [1–4]. However, there have been few investigations on the bioprocess development of mushroom submerged fermentation [1,2].

Cordyceps militaris, a caterpillar-shaped Chinese traditional medicinal mushroom, is an entomopathogenic fungus, which belongs to the class Ascomycetes and DongChongXiaCao group in Chinese herbs [5]. Besides its usage as a crude drug, it has been extensively used as folk tonic food or invigorant since ancient times [5,6]. This mushroom produces an important bioactive compound, cordycepin (3′-deoxyadenosine), which is a nucleoside analogue [7,8]. Cordycepin is reported to possess many interesting biological and pharmacological activities, including immunological stimulating, anti-cancer, anti-virus and anti-infection activities [7–11]. Previous work reported the isolation of cordycepin from liquid culture medium of *C. militaris* and its pharmacological functions [7,8,12–13]. Trace levels of cordycepin were mentioned in mycelium and culture broth during submerged cultivation of *Cordyceps* sp. in potato-dextrose medium [14]. Jia et al. reported that 7.1 mg cordycepin/l was acquired in an airlift bioreactor cultivation of *C. militaris* [15]. However, there have been no reports on dynamic profiles of cordycepin production by submerged cultivation of *C. militaris*, and the effect of medium nutrients has not been revealed as yet.

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Usually, culture medium is important to the yield of any fermentation products, and carbon and nitrogen sources generally play a significant role because these nutrients are directly linked with cell proliferation and metabolite biosynthesis [1–4,16]. Various statistical experimental design strategies were applied to the optimization of fermentation media such as for lovastatin production by *Monascus rubber* [17]. But, as far as we know, there is limited knowledge about the nutritional requirement for cordycepin production by *C. militaris*, and there have been no reports on medium optimization to improve cordycepin production. In this work, the effects of carbon sources and carbon/nitrogen ratios were focused in order to improve the cordycepin production by submerged cultivation of *C. militaris*. The information obtained is considered fundamental and useful to the development of *C. militaris* cultivation process for efficient production of cordycepin on a large scale.

2. Materials and methods

2.1. Maintenance and seed culture of C. militaris

The strain of *C. militaris* was purchased from the collection bank of Huazhong Agricultural University (Hubei, China). The stock culture was maintained on potato-dextrose-agar slants. The slants were inoculated with mycelia and incubated at 25 °C for 7 days, and then used for seed culture inoculation. The seed culture medium consisted of the following components: glucose, 40 g/l; yeast extract, 10 g/l; KH_2PO_4, 0.5 g/l; $K_2HPO_4{\cdot}3H_2O$, 0.5 g/l and $MgSO_4{\cdot}7H_2O$, 0.5 g/l. The mycelia of *C. militaris* were transferred to the seed culture medium by punching out about 5 mm^2 of the slants with a sterilized cutter. The seed culture was grown in a 250 ml shake flask containing 50 ml of liquid medium and incubated at 25 °C on a rotary shaker (110 rpm) for 5 days.

2.2. Experiments on carbon source, initial glucose level and carbon/nitrogen ratio

Effects of carbon sources on liquid culture of *C. militaris* were studied using 40 g/l of one of the following carbon sources, i.e. lactose, sucrose, glucose, fructose, galactose, maltose and xylose. The other culture medium components were 10 g/l of peptone, 0.5 g/l of KH_2PO_4, 0.5 g/l of $K_2HPO_4{\cdot}3H_2O$ and 0.5 g/l of $MgSO_4{\cdot}7H_2O$. For the investigation on initial sugar concentrations, glucose was used with levels of 25, 40, 55 and 70 g/l tested. In experiments on effects of carbon/nitrogen ratios, the levels of glucose and peptone in medium were changed and a statistical approach was applied. Inoculation was done by transferring 5 ml of the above seed culture medium (with ca. 12 g DW/l) to 45 ml medium in a 250 ml shake flask. The cultivation was conducted at 25 °C on a rotary shaker (110 rpm). In all experiments, multiple flasks at least in duplicate were run at the same time to ensure reproducibility.

2.3. Central composite design

Central composite design (CCD) was conducted in the optimum vicinity to locate the true optimum concentrations of glucose and peptone for cordycepin production [17]. The levels of variables for CCD experiments were selected according to the results of one-at-a-time strategy. The CCD experimental results were fitted with a second-order polynomial equation of Eq. (1) by a multiple regression technique.

$$y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (1)$$

where y is predicted response, β is constant coefficient, and x is the coded independent variable.

The fitness of the second-order model was expressed by the regression coefficient R^2 and its statistical significance was determined by an F-test. The regression significance was tested by a t-test. The SAS software (version 8.0 by SAS Institute Inc., NC, USA) and MatLab software (version 6.5 by the Mathworks, Inc.) were used for regression and graphical analyses of the data obtained, respectively.

2.4. Analytical procedures

2.4.1. Determination of dry cell weight (DW), pH and residual medium sugar

For the measurement of DW, the cells from a sample were filtered through a filter paper and washed twice with distilled water. The fresh cells were dried at 60 °C for sufficient time until a constant DW was obtained. After sampling, one part of the culture filtrate was used for measuring pH value with a pH meter; another part was stored at −20 °C, and later thawed for analyses of residual sugar and cordycepin production. Residual sugar concentration was assayed by a phenol–sulphuric acid method [18].

Average growth rate was calculated as: (maximum DW – initial DW)/[(initial DW)(culture time)] (unit: day^{-1}), and growth yield on sugar was calculated as: (maximum DW – initial DW)/(initial sugar concentration – residual sugar concentration)(unit: g DW/g sugar).

2.4.2. Cordycepin production [8,15]

Standard cordycepin (from Sigma) was dissolved in distilled water for calibration. The cordycepin concentration was analyzed by HPLC. A column Kromasil C_{18} (4.6 mm × 250 mm, 5 μm particle size) (Eliter Company, Liaonin, China) was used. The mobile phase was 10 mM KH_2PO_4, which was dissolved in methanol/distilled water (15:85) and the mobile phase was driven by double pump (model: Waters 150, Millipore, USA). Elution was performed at a flow rate of 1 ml/min with column temperature at 30 °C. The UV