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Original Article

Optimization of fermentation process of *Cordyceps militaris* and antitumor activities of polysaccharides *in vitro*

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ABSTRACT

The influence of medium composition and cultural conditions on simultaneous yield of mycelia, intracellular polysaccharide, adenosine, and mannitol by *Cordyceps militaris* CGMCC 2909 was investigated with desirability functions in this study. An optimization strategy based on the desirability function approach, together with response surface methodology (RSM) has been used to optimize medium composition, and the optimal medium was obtained via the desirability as follows: yeast extract 10.33 g/L, sucrose 27.24 g/L, KH_2PO_4 5.60 g/L and the optimal culture conditions are initial pH 6, 25°C, rotation speed 150 r/minute, inoculum size 4%(v/v), and medium capacity 40 mL/250 mL. Under these conditions, the yield of mycelia, intracellular polysaccharide, adenosine and mannitol reached 12.19 g/L, 0.6 g/L, 61.84 mg/L, and 1.38 g/L, respectively, and the *D* value was 0.77. Furthermore, the polysaccharides showed significant antitumor activities against HeLa and HepG2 *in vitro* in a dose-dependent manner in 72 hours. At a concentration of 1000 mg/mL, the inhibition rate of polysaccharides was 92.38% and 98.79%. The IC50 for HeLa and HepG2 were 70.91 μg/mL and 97.63 μg/mL, respectively.

1. Introduction

Medicinal mushrooms are an abundant source of a wide range of useful natural products with biological activities. *Cordyceps militaris*, one of the Chinese traditional medicinal mushrooms, is an entomopathogenic fungus belonging to the class Ascomycetes. It has been widely used as folk tonic food and an invigorant for centuries and its various pharmacological activities have attracted much attention [1]. This mushroom produces many kinds of active components such as adenosine, polysaccharides, and mannitol [2–8]. Adenosine has many pharmacological effects; it can be used as a cardioprotective and therapeutic agent for chronic heart failure [9], and it also could inhibit the release of neurotransmitters in the central nervous system [10]. Polysaccharides are considered to possess antiinflammatory, antioxidant, antitumor, antimetastatic, immunomodulatory,

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hypoglycemic, steroidogenic, and hypolipidemic effects [11–17]. Mannitol (cordycepic acid) has diuretic, antifree radical, and antitussive activities, etc. [18].

Since solid culture of fungi takes a longer time to yield the fruiting body with slight production of an active substance, submerged cultivation of fungi is considered as a promising alternative for obtaining useful and potent substances for use in the composition of nutraceuticals and functional foods [19]. How to shorten the fermentation time of a fungus and increase the production of fermentation is still a challenge. Mycelium and biological activity metabolites were mainly used as a single response to optimize submerged cultivation [20,21]; however, two or multiple responses have already been used in the process optimization [22–24]. There was not, however, a solution to the problem of simultaneously enhancing the production of multiple responses in *C. militaris* submerged cultivation. Response surface methodology (RSM) is a collection of statistical and mathematical techniques and has been proven to be an effective mean, which includes studying the response of the statistically designed combinations, estimating the coefficients by fitting in a mathematical model which fits best the experimental conditions, predicting the response of the fitted model, and checking the adequacy of the model.

In recent years, studies of antitumor activities of *C. militaris* have been of particular interest. In a study by Park et al [25], an aqueous extract of *C. militaris* could inhibit cell growth of human leukemia U937 cells, by morphological change and apoptotic cell death. In a study by Yoo et al [26], *C. militaris* extract could reduce angiogenic related gene expression and inhibit cell growth of B16 melanoma cells. Cordycepin and polysaccharides, as the main constituents of *Cordyceps* species, are detected to have cytotoxic and antitumor activity [27–30].

In this study, we investigated the medium composition and cultural conditions for the submerged culture of *C. militaris* 2909 to obtain optimal production of mycelia, polysaccharides, adenosine, and mannitol, using sequential statistical methods combined with desirability function at the same time. In addition, the growth inhibitory effect of *C. militaris* polysaccharides on HeLa and HepG2 cells was studied.

2. Materials and methods

2.1. Microorganism and seed culture

The strain of *C. militaris* (CGMCC 2909) was maintained and cultured on potato-dextrose-agar slants, incubated at 25°C for 7 days, and then used for seed culture inoculation. The seed culture medium consisted of the following components: sucrose 25 g/L, peptone 10 g/L, yeast extract powder 20 g/L, KH_2PO_4 3 g/L, $MgSO_4 \cdot 7H_2O$ 3 g/L, $(NH_4)_2SO_4$ 10 g/L, $ZnCl_2$ 0.01 g/L, and vitamin B1 0.24 g/L. The fermentation medium was based on the seed culture medium to monitor carbon and nitrogen sources, mineral elements, etc. The flask culture incubated in a 250 mL Erlenmeyer flask containing 100 mL of the medium after inoculation with 2% (v/v) of the seed culture at 25°C on a rotary shaker incubator at 150 rpm for 5 days.

2.2. Analytical methods

2.2.1. Determination of mycelia dry weight

Mycelia of *C. militaris* in the fermentation broth were centrifuged, the supernatant was filtered through a filter paper and washed twice with distilled water, transferred to a lyophilizer *in vacuo*, dried to a constant weight and recorded as the dry weight, and then homogenized to powders (80 mesh).

2.2.2. Measurements of polysaccharides

Dried mycelia powders were extracted three times with distilled water for 3.5 hours in an 80°C water bath (mycelia/distilled water ratio, 1:5), and then centrifuged at 7000*g* for 10 minutes. The amount of polysaccharides was then determined by a phenol-sulfuric acid method.

2.2.3. Measurement of adenosine by high performance liquid chromatography

An amount of 0.1 g dried mycelia powders was extracted with 5 mL distilled water for 3 hours in a 45°C water bath, and then centrifuged at 12,000*g* for 10 minutes. The supernatant was filtered with a 0.45 μm membrane, and the filtrate was analyzed by high performance liquid chromatography. A Kromasil C18 column (4.6 mm × 250 mm, 5 μm particle size) (Eliter Company, Liaoning, 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 a double pump (model: Waters 150, Millipore, Bedford, USA). Elution was performed at a flow rate of 1 mL/minute with a column temperature of 30°C. The UV wavelength of 260 nm was monitored by a tunable absorbance detector (model: Waters 486, Millipore). A 20 μL syringe was used for injection.

2.2.4. Determination of mannitol

Dried mycelia powders (0.1 g) were extracted with 5 mL distilled water for 3 hours in a 45°C water bath, and then centrifuged at 8000*g* for 10 minutes. The supernatant was diluted 50 times. The amount of mannitol was then determined by the spectrophotometric method [31].

2.2.5. Desirability

In the desirability function approach, the multicriteria problem is reduced to a single criterion problem of *D* optimization. In the present study, the yield of mycelia, polysaccharides, adenosine, and mannitol were considered as four quality variables. The higher yields of the four variables were sought, which include the most desirable (y_{max}) and the undesirable values (y_{min}). The measured properties of each predicted response *y* are transformed to a dimensionless desirability value *d*. The scale of value *d* ranges between 0 and 1, and becomes more desirable as the corresponding response value increases. The desirability function contributed by the response is defined as follows:

$$\begin{aligned} &d = 0,\ y < y_{min} \\ &d = \frac{y - y_{min}}{y_{max} - y_{min}},\ y_{min} \le y \le y_{max} \\ &d = 1,\ y > y_{max} \end{aligned} \quad (1)$$

where *d* is the desirability. The desirability represents the closeness of a response (*y*) to its most desirable value (y_{max}),