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Application of Box-Behnken design in optimisation for polysaccharides extraction from cultured mycelium of *Cordyceps sinensis*

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ABSTRACT

A three-level Box-Behnken design, combined with the canonical and ridge analyses, was employed to optimise the process parameters for polysaccharide extraction from cultured mycelium of *Cordyceps sinensis*, one of the most valued traditional Chinese medicines and health foods. The critical factors selected for the investigation were extraction temperature, duration of time and number of times. The experimental results were fitted with a second-order polynomial equation by a multiple regression analysis and more than 96% of the variation could be predicted by the models. The canonical analysis of surface responses revealed that the three eigenvalues had different signs, indicating a saddle stationary surface. The optimal conditions for extraction of polysaccharides from the cultured mycelium of *C. sinensis* were determined, using the ridge analysis, as extracting 110 min at 88.9 °C for three times. Under the optimal conditions the corresponding response value predicted for polysaccharide production was 15.85%, which was confirmed by validation experiments.

Keywords: *Cordyceps sinensis*; Polysaccharides; Box-Behnken design; Canonical analysis; Ridge analysis

1. Introduction

Cordyceps sinensis (Berk.) Sacc. is an entomogenous fungus belonging to *Ascomycota*, *Sordariomycetes*, *Hypocreales*, *Clavicipitaceae* (Lindau) O.E. Erikss., *Cordyceps* (Fr.) Link (Kirk et al., 2001). It is popularly referred to as the Chinese Caterpillar Fungus or 'Dong Chong Xia Cao' summer-plant, winter-worm) in Chinese. Early English translations of 'Hia Tsao Tong Tchong' and 'Hea Tsaon Tsong Chung' are also found in the western literature (Pegler et al., 1994). As a traditional Chinese medicine and health foods, *C. sinensis* is considered to have the similar medicinal effects of ginseng and deer velvet. It has been used in China for thousands of years and has been regarded as a celebrated drug in the Chinese Pharmacopeia since 1963 (CPCMH, 1964). It has been used to treat a wide range of conditions, including respiratory, liver and cardiovascular diseases, hypo-sexuality and hyperlipidemia.(Wang, 1995; Zhu et al., 1998). However, the natural resources of *C. sinensis* are very limited due to its confined geographic distribution on the Tibetan Plateau and over exploitation in recent years. For alternatives, culture of the fungus in submerged fermentation to produce mycelium in large quantity has been proved as a promising way to meet the needs of human consumption and to reduce the pressure on natural resources of the species which is in danger (Yao, 2004).

Many biologically active polysaccharides have been isolated from various fungi and some of them are now used in clinics, e.g. lentinan from *Lentinula edodes* (Berk.) Pegler (Zheng et al., 2005), schizophyllan from *Schizophyllum commune* Fr. (Krosl and Korbelik, 1994; Kubala et al., 2003) and protein-bound polysaccharide K (PSK) from *Coriolus versicolo* (L.) Quél. (Matsunaga et al., 1998; Kanazawa et al., 2004). These

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polysaccharides have been shown to enhance and stimulate the immune system of human and of mice, and are thus called biological response modifiers (Wasser, 2002).

Polysaccharides from *C. sinensis* have been demonstrated to have many biological activities, e.g. antitumour (Yang et al., 2005), antioxidation (Li et al., 2003), hypoglycaemic (Kiho et al., 1999) and hypocholesterolemic effects (Kiho et al., 1996). As polysaccharides are mainly present in the cell wall and within cytoplasm, improvement of the extraction rate of polysaccharides from cultured mycelium is evidently important to the efficiency of extraction process. Sun et al. (2003) studied the effects of granularity of mycelial powder, ratio of water to mycelium, duration of time of extraction and pH on the yield of polysaccharides with the one-variable-at-a-time method, for a so-called *C. sinensis* strain named '*Paecilomyces hepiali* chen et dai sp nov' [*sic*!] (An invalid fungal name, see Jiang and Yao (Jiang and Yao, 2002, 2003). It is clearly not a strain of real *C. sinensis*). Yu et al. (2002) optimised the extraction parameters, including duration of time of ethanol precipitation, the ratio of extraction solvent to fermentation broth and pH for the polysaccharide extraction from fermentation broth of *C. sinensis*. Numerous papers have been published in regard to polysaccharides from this fungus (Gong et al., 1990; Li et al., 2003; Wu et al., 2005), but none was on the optimisation of process parameters for polysaccharide extraction from the cultured mycelium of *C. sinensis*.

The response surface methodology (RSM) is a collection of mathematical and statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum condition of factors for desirable responses (Box et al., 1978). The optimisation process of this methodology involves studying the response of the statistically designed combinations, estimating the coefficients by fitting it in a mathematical model that fits best the experimental conditions, predicting the response of the fitted model and checking the adequacy of the mode. The most common designs, i.e. central composite design (CCD) and Box-Behnken design (BBD), of the principal response surface methodology have been widely used in various experiments (Box et al., 1978; Dean and Voss, 1999). Box-Behnken, a spherical and revolving design, has been applied in optimisation of chemical and physical processes (Oscar et al., 1999; Qiu and Chen, 1999; Muthukumar et al., 2003) because of its reasoning design and excellent outcomes.

In the present work, Box-Behnken design, followed by canonical and ridge analyses, was employed to optimise the process parameters of polysaccharide extraction from the cultured mycelium of *C. sinensis* so as to facilitate the further and reasonable exploration of this treasured fungus.

2. Materials and methods

2.1. Fungal strain

The strain, No. 762, used in this study was originally isolated from *C. sinensis* collected from Sichuan, China by this laboratory. It was maintained on potato dextrose agar (PDA) supplemented with 5% wheat bran and 0.5% peptone at 4 °C. The Internal Transcribed Spacer (ITS1-5.8S-ITS2) of nuclear ribosomal DNA (nrDNA) was amplified from the culture and 496 bp of the fragment were obtained from DNA sequencing. The sequence was compared with a data set generated in this laboratory containing ITS sequences from dried specimens and living strains of *C. sinensis* obtained from various regions of the Tibetan Plateau to confirm the identity of the strain.

2.2. Inoculum preparation and submerged culture

The strain was first incubated on the same medium as for the stock at 18 °C for 45 d in Petri dish and then transferred to 500 ml Erlenmeyer flasks with the same medium without agar (Dong and Yao, 2005) by punching a 5-mm agar disc from the 45-d culture with a sterilized cutter. The flasks, containing 100 ml of liquid medium, were rotated at 100 rpm, at 18 ± 1 °C for 45 d.

The mycelium was harvested by centrifugation for 15 min at 8000 × *g* to separate it from the liquid medium. After repeated washing with distilled water, the mycelial pellets were lyophilized using a VirTis freeze dryer (VirTis Co., Gardiner, New York) for later experiments.

2.3. Extraction of mycelial polysaccharides

The powder of lyophilized mycelium (1.000 g) was extracted several times with 20 volumes of distilled water at 70–90 °C for 1–3 h each time. After vacuum filtration, the aqueous extracts were combined and concentrated to one-third of its total volume in vacuum. The resulting concentrated liquor was mixed with three times of its volume of absolute ethanol, stirred vigorously and left overnight at 4 °C. The precipitated polysaccharides were centrifuged at 8000 × *g* for 30 min and the supernatant discarded. The precipitate of crude polysaccharides was dried at 65 °C to a constant weight and the polymer was weighted by a scale (Sartorious ALC-110.4, Germany). The product yield was measured at the (w/w) % of polysaccharides per unit mass of lyophilized mycelium.

2.4. Box-Behnken design

According to the principle of Box-Behnken design, extraction temperature, duration of time and number of times, which were identified to have strong effects on the response in preliminary one-factor-at-a-time experiments, were taken as the variables tested in a 15-run experiment to determine their optimum levels. As shown in Table 1, the three factors chosen for this study were designated as X_1, X_2, X_3 and prescribed into three levels, coded +1, 0, −1 for high, intermediate and low value, successively. Three test variables were coded according to the following equation:

$$x_i = \frac{X_i - X_0}{\Delta X} \quad i = 1, 2, 3$$

where x_i is the coded value of an independent variable; X_i is the actual value of an independent variable; X_0 is the actual value of an independent variable at centre point; and ΔX is the step change value of an independent variable. Table 2 shows the Box-Behnken design matrix of the experiment of 15 trials. All experiments were performed in triplicate and the averages of polysaccharide yield were taken as response.

For predicting the optimal point, a second-order polynomial model was fitted to correlate relationship between independent variables and response (polysaccharide yield). For the three factors, the equation is

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2$$