Chiang Mai J. Sci. 2016; 43(5) : 972-984
http://epg.science.cmu.ac.th/ejournal/
Contributed Paper

# Enhanced Production of Cordycepin by Solid State Fermentation of *Cordyceps militaris* using Additives

**Ting-Chi Wen [a]+, Chao Kang [b]+, Ze-Bing Meng [a], Yong-Bin Qi [a], Kevin D. Hyde [c, d] and Ji-Chuan Kang* [a]**

[a] The Engineering and Research Center of Southwest Bio-Pharmaceutical Resources Ministry of Education, Guizhou University, Guiyang 550025, Guizhou Province, P.R. China.

[b] Institute of Biology, Guizhou Academy of Sciences, Guiyang, 550009, Guizhou Province, P.R. China.

[c] Institute of Excellence in Fungal Research, and School of Science, Mae Fah Luang University, Chiang Rai 57100, Thailand.

[d] Botany and Microbiology Department, College of Science, King Saud University, Riyadh 11442, Saudi Arabia.

+ These authors contributed equally to this work.

*Author for correspondence; e-mail: bcec.jckang@gzu.edu.cn

Received: 10 July 2015
Accepted: 8 March 2016

## ABSTRACT

Cordycepin is one of the most important bioactive compounds produced by the insect pathogenic fungus *Cordyceps militaris*. The effects of medium composition on enhanced production of cordycepin by *C. militaris* were investigated by changing the liquid supplement medium composition with rice medium in this study. Glucose, peptone, adenine, and histidine were optimized to improve the yield of cordycepin in 300 mL cylindrical glass bottles by solid state fermentation using response surface methodology. The optimum medium for cordycepin production using solid state fermentation were: glucose 26.25 g/L, peptone 26.25 g/L, adenine 7.50 g/L, histidine 4.50 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, and $KH_2PO_4$ 0.1 g/L in liquid supplement medium. Under these conditions, the maximum cordycepin yield in the rice culture medium was 18.92 mg/g on 39 days, and this value is higher when compared to other reports using solid state fermentation. This is the first report for improving the cordycepin production by adding additives (nucleoside analogue and amino acid) in solid state fermentation by *C. militaris*. This method provides an effective way for increasing the cordycepin production by solid state fermentation.

**Keywords:** *Cordyceps militaris*, cordycepin, solid state fermentation, additives, response surface methodology

## 1. INTRODUCTION

*Cordyceps militaris* has long been used as a medicinal mushroom in China and East Asia [1]. *C. militaris* has long been recognized as a desirable alternative for natural *Ophiocordyceps sinensis* [2] as has been given Chinese Licence No: Z20030034/35. This

is because the gathering of *O. sinensis* is causing substantial reductions in populations [3]. *C. militaris* possesses many biological and pharmacological active substances such as adenosine, cordycepin, amino acid and fatty acids [4]. The effective components of *C. militaris* were obtained in fermentation broth and/or from fruiting body in submerged or solid-state cultures.

Expressly, Cordycepin (3′-deoxyadenosine), a nucleoside analog, was first isolated from *C. militaris* [5] , and has a broad spectrum of biological activity. It has been regarded as a medicinal agent responsible for immunological regulation [6], anti-cancer [6], anti-fungal [7], anti-leukemia [1] and anti-hyperlipidemia [8] activities. Cordycepin is also a Phase I/II clinical stage drug candidate for treatment of Refractory Acute Lymphoblastic Leukemia (ALL) patients who express the enzyme terminal deoxynucleotidyl transferase (TdT) (www. ClinicalTrials. Gov, verified by OncoVista, Inc., 2009).

Fruiting body formation of *Cordyceps* needs a long cultivation time [9]. Furthermore, repeated subculturing results in strains of *C. militaris* producing fewer or no fruiting bodies [9]. A research shows that $F_1$ progeny strains of *C. militaris* generally produce a larger number of fruiting bodies, as compared with their mother multi-ascospore isolates; however, $F_2$ and $F_3$ progeny produce fewer fruiting bodies [10]. The extraction of cordycepin from fruiting bodies of *C. militaris* is also difficult. However, large quantities of the cordycepin are secreted into culture medium [11]. In previous work, cordycepin was obtained from fruiting bodies via solid-state fermentation [12] and fermentation broth by submerged culture [13, 14] or surface liquid culture [15]. However, there is no report for improving the cordycepin production by adding additives (nucleoside analogue and amino acid) in solid state fermentation by *C. militaris*.

In this paper, the effects of glucose, peptone, nucleoside analogue and amino acid were studied in order to improve the cordycepin production of *C. militaris* CM016 in the rice medium by solid state fermentation in 300 mL cylindrical glass bottle. This is also the first report for improving the cordycepin production by adding additives (nucleoside analogue and amino acid) in solid state fermentation by *C. militaris*.

## 2. MATERIALS AND METHODS

### 2.1 Microorganism and Medium

The strain of *C. militaris* CM016 was purchased from the Collection Center of Beijing Jixiaoyuan Co. Ltd. (Beijing, China). The stock culture was maintained on potato dextrose agar (PDA) slants. The slants were inoculated with mycelia and incubated at 25°C for 7 days, and 6 ml sterilized distilled water was added to the slant and the conidia were washed down and then filtered through sterilized absorbent cotton in an infundibulum. The liquid filtrate containing conidia was added to seed culture medium at a suitable concentration (optimal concentration is $3\times10^8$ conidia/ml, the number of conidia was counted using a hematocytometer). The seed culture medium consisted of the following components: sucrose, 20 g/L; peptone (BWC022, Bio-way, Shanghai), 20 g/L; MgSO4·7$H_2O$ 0.5 g/L and $K_2HPO_4$, 1g/L. All chemicals and reagents were of analytical grade. 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 (150 rpm) for 4 days [12, 16].

### 2.2 Solid-state Fermentation

The medium for solid state fermentation was prepared by mixing 20 g of rice (*Oryza*

*sativa* L. ssp. indica) and 32 mL of the basal liquid supplement medium (sucrose, 20 g/L; peptone, 10 g/L; $MgSO_4 \cdot 7H_2O$ 0.1 g/L and $KH_2PO_4$ 0.1 g/L with 1000 mL distilled water) in a 300 mL cylindrical glass bottle (inner diameter 7 cm, height 12 cm) and the bottle was sealed by film of polypropylene plastic. The medium was autoclaved for 20 min at 121°C. The medium was cooled to room temperature and inoculated with 5 ml seed culture and incubated at 20 °C for 12 days in the dark to promote vegetative growth.

The incubation temperature was raised to 23°C, light maintained at 500 lx for 12 hours one day and relative humidity (RH) at 60%-70% for 12-20 days. Sufficient air changes were made to maintain a normal $CO_2$ level. Illumination with 300 lx intensity did not exceed 12 hours per day for the whole remain fermentation process [9].

### 2.3 Response Surface Methodology Design

Response surface methodology (RSM) using a central composite design (CCD) was applied to batch cultures of *C. militaris*, for identifying the effects of process variables (glucose, peptone, adenine, histidine). A four-factor, five-level central composite design with 30 runs was employed. Tested variables (glucose, peptone, adenine, histidine) were denoted as $X_1$, $X_2$, $X_3$ and $X_4$, respectively, and each of them was assessed at five different levels, combining factorial points (-1, +1), axial points (-2, +2), and central point (0), as shown in Table 1.

**Table1.** Factors and levels of central composite design for glucose, peptone, adenine, histidine.

| Independent variables | Symbols | Code Levels | | | | |
|---|---|---|---|---|---|---|
| | | -2 | -1 | 0 | +1 | +2 |
| Glucose (g/L) | $X_1$ | 17.5 | 26.25 | 35 | 43.75 | 52.5 |
| Peptone (g/L) | $X_2$ | 17.5 | 26.25 | 35 | 43.75 | 52.5 |
| Adenine (g/L) | $X_3$ | 3 | 4.5 | 6 | 7.5 | 9 |
| Histidine (g/L) | $X_4$ | 3 | 4.5 | 6 | 7.5 | 9 |

### 2.4 Analytical Methods

For analysis of total cordycepin, adenine and adenosine in the medium (contain rice substrate and mycelium of *C.militaris*), the fresh medium collected from the cylindrical glass bottle were dried to a constant weight at 55°C. The samples were crushed with a grinder and then sieved (60 meshes). The crushed sample (0.1g) was added to the glass tube containing 10 mL of distilled water, and treated by ultrasonic wave for 30 minutes, and diluted 10 times with distilled water. The water solution was filtered through a 0.45 μm membrane and the filtrate was analyzed by HPLC (1100 series, Agilent Technology, U.S.). Accurate quantities of cordycepin (Sigma, USA), adenine (Sigma, USA) and adenosine (Sigma, USA ) were dissolved in distilled water, to give various concentrations for calibration. The mobile phase was 10 mmol/L $KH_2PO_4$, which was dissolved in methanol/distilled water (6:94). Elution was performed at a flow rate of 1 mL/min with column temperature at 45°C and UV wavelength of 259 nm [15]. All experiments were triplicated.

### 2.5 Statistical Analysis

Data were expressed as mean ± SD. Duncan's multiple range tests, at $P<0.05$ and $P<0.01$, found significant differences among means using the SPSS software 17.0 (SPSS Inc., Chicago, IL, USA). RSM and analysis of variance (ANOVA) were performed using the Design-expert Version 8.0.5b software package (Stat-Ease Inc., Minneapolis, USA). All data presented are mean values of three determinations.

## 3. RESULTS

### 3.1 Cordycepin Production in the Basal Medium

To confirm a suitable cultivation time for cordycepin production by solid state fermentation, *C. militaris* was cultivated in a 300 ml cylindrical glass bottle under the basal medium. The result (Figure 1) showed that cordycepin production gradually increased with the cultivation time, and a maximal cordycepin production was 3.3 ± 0.03 mg/g on 39 days. However, cordycepin production reduced gradually with increasing cultivation time from 39 to 65 days. Therefore, the cultivation time of 39 day was selected as the standard cultivation period in the remaining experiment.

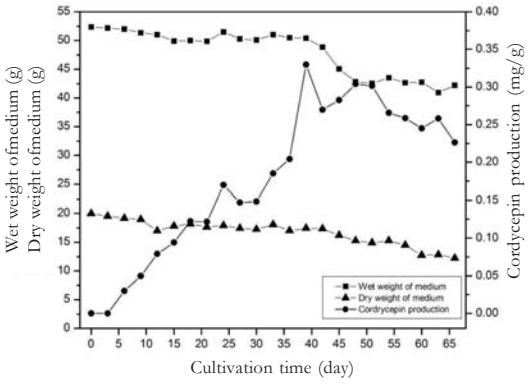


**Figure 1.** Changes of cordycepin production (%), wet weight of medium (%), and dry weight of medium (%) in the basal medium by solid-state fermentation in *C. militaris*.

### 3.2 Effect of Nucleoside on Cordycepin Production

In order to identify a suitable nucleoside for cordycepin production by solid state fermentation of *C. militaris*, different nucleosides (adenine, guanine, guanosine, inosine, uridine, thymine, cytosine, hypoxanthine, adenosine, thymidine and deoxyuridine) were added to the basal liquid supplement medium at a concentration of 2 g/L. As shown in Table 2, all nucleoside could increase the yield of cordycepin. Adenine, cytosine and thymidine can obviously improve cordycepin production at 5.64 ± 1.20 mg/g, 5.59 ± 0.72 mg/g and 5.91 ± 0.53 mg/g respectively. There was no significant difference in the amount of adenine and adenosine produced as compared with the control.

### 3.3 Selection of Initial Nucleoside Concentrations

Based on the above results, adenine, cytosine, and thymidine were suitable nucleosides for further studies. Table 3 summarizes the effect of initial adenine, cytosine, and thymidine concentrations on the wet weight of medium, dry weight of medium, adenine, adenosine, and cordycepin production. The highest of cordycepin production (9.98 ± 0.27 mg/g) was obtained at 6 g/L initial thymidine. It can be seen from the comparison in Table 3 that cordycepin production showed third higher yields (8.54 ± 0.50 mg/g) when adenine was added at 6 g/L. However, the cost of thymidine is much higher than adenine, and there was no significant difference between thymidine and adenine, so adenine was chosen.

**Table 2.** Effects of nucleosides on the production of cordycepin, adenine and adenosine.

| Nucleoside | Wet weight of medium (g) | Dry weight of medium (g) | Adenine (mg/g) | Adenosine (mg/g) | Cordycepin (mg/g) |
|---|---|---|---|---|---|
| Control | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| Adenine | 46.68±0.08 | 9.08±0.27 | 0.03±0.00 | 0.36±0.06 | 5.64±1.20 |
| Guanine | 48.66±0.61 | 10.47±0.6 | 0.03±0.00 | 0.22±0.04 | 4.01±1.23 |
| Guanosine | 45.33±0.37 | 10.0±0.24 | 0.04±0.01 | 0.29±0.04 | 2.57±0.50 |
| Inosine | 47.29±0.75 | 9.64±0.11 | 0.05±0.02 | 0.50±0.05 | 3.79±0.19 |
| Uridine | 47.19±0.64 | 9.59±0.29 | 0.03±0.00 | 0.43±0.10 | 4.06±0.42 |
| Thymine | 44.97±0.12 | 8.74±0.04 | 0.07±0.01 | 0.68±0.00 | 4.50±0.69 |
| Cytosine | 45.55±0.82 | 9.55±0.57 | 0.11±0.01 | 0.67±0.12 | 5.59±0.72 |
| Hypoxanthine | 43.59±0.62 | 10.33±0.20 | 0.05±0.02 | 0.63±0.17 | 5.57±1.14 |
| Adenosine | 43.99±0.65 | 8.99±0.57 | 0.04±0.01 | 0.21±0.08 | 5.53±1.00 |
| Thymidine | 47.78±0.28 | 8.98±0.19 | 0.04±0.01 | 0.53±0.15 | 5.91±0.53 |
| Deoxyuridine | 50.19±0.74 | 8.59±0.72 | 0.05±0.01 | 0.15±0.03 | 5.14±0.98 |

**Table 3.** Effects of initial nucleoside concentrations on the production of cordycepin, adenine and adenosine.

| Nucleoside | Concentration (g/L) | Wet weight of medium (g) | Dry weight of medium (g) | Adenine (mg/g) | Adenosine (mg/g) | Cordycepin (mg/g) |
|---|---|---|---|---|---|---|
| Adenine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 53.17±0.14 | 15.66±0.18 | 0.22±0.01 | 0.14±0.02 | 8.30±0.86 |
| | 1 | 50.79±0.27 | 15.66±0.16 | 0.10±0.01 | 0.15±0.03 | 5.42±0.29 |
| | 2 | 50.79±0.81 | 14.96±0.08 | 0.07±0.02 | 0.11±0.04 | 7.04±0.53 |
| | 4 | 52.10±0.64 | 16.11±0.10 | 0.22±0.04 | 0.17±0.04 | 7.27±0.24 |
| | 6 | 49.41±0.28 | 15.89±0.16 | 0.56±0.06 | 0.32±0.09 | 8.54±0.50 |
| | 8 | 51.09±0.48 | 16.39±0.21 | 0.86±0.09 | 0.27±0.11 | 7.36±0.08 |
| | 10 | 52.72±0.57 | 16.54±0.15 | 0.37±0.06 | 0.16±0.04 | 7.67±0.40 |
| Cytosine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 48.01±0.88 | 14.51±0.13 | 0.09±0.03 | 0.06±0.00 | 6.63±0.23 |
| | 1 | 51.78±0.20 | 15.43±0.28 | 0.03±0.01 | 0.17±0.00 | 2.64±0.50 |
| | 2 | 49.37±0.83 | 14.37±0.19 | 0.03±0.01 | 0.07±0.04 | 2.40±0.28 |
| | 4 | 48.83±0.56 | 13.90±0.11 | 0.05±0.01 | 0.08±0.00 | 3.09±0.50 |
| | 6 | 51.91±0.76 | 16.07±0.11 | 0.04±0.01 | 0.21±0.05 | 3.12±0.72 |
| | 8 | 50.74±0.01 | 16.53±0.42 | 0.05±0.03 | 0.15±0.00 | 2.88±0.56 |
| | 10 | 51.95±0.59 | 16.63±0.11 | 0.08±0.00 | 0.18±0.05 | 4.69±0.72 |
| Thymidine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 48.01±0.28 | 12.46±0.46 | 0.05±0.02 | 0.11±0.01 | 3.59±0.11 |
| | 1 | 50.63±0.07 | 15.45±0.07 | 0.04±0.00 | 0.13±0.01 | 9.48±0.57 |
| | 2 | 50.85±0.39 | 15.73±0.37 | 0.03±0.00 | 0.19±0.00 | 7.93±0.28 |
| | 4 | 52.94±0.41 | 15.33±0.30 | 0.17±0.00 | 0.05±0.00 | 7.68±0.10 |
| | 6 | 49.99±0.66 | 13.39±0.28 | 0.04±0.00 | 0.05±0.00 | 9.98±0.27 |
| | 8 | 48.74±0.03 | 16.75±0.15 | 0.08±0.00 | 0.10±0.00 | 5.11±0.66 |

### 3.4 Effect of Amino Acids

In order to identify a suitable amino acid for cordycepin production by solid state fermentation of *C. militaris*, different amino acids (glutamic acid, cysteine, lysine, isoleucine, methionine, tyrosine, cystine, L-hydroxyproline, valine, histidine, arginine, tryptophan, leucine, alanine, proline, phenylalanine, asparagine, glycine, aspartate acid, and glutamine) were added to the basal liquid supplement medium at a concentration of 4 g/L. As shown in Table 4, most amino acids (except methionine and tyrosine) can increase the yield of cordycepin production (Table 4). Among various kinds of amino acids, lysine, histidine and glycine greatly improved cordycepin production at 7.18 ± 1.12 mg/g, 6.95 ± 1.01 mg/g, 7.08 ± 0.90 mg/g. respectively.

**Table 4.** Effects of amino acids on the production of cordycepin, adenine and adenosine.

| Amino acid | Wet weight of medium (g) | Dry weight of medium (g) | Adenine (mg/g) | Adenosine (mg/g) | Cordycepin (mg/g) |
|---|---|---|---|---|---|
| Control | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| Glutamic acid | 46.92±0.25 | 9.30±0.21 | 0.02±0.00 | 0.28±0.02 | 5.58±0.72 |
| Cysteine | 49.43±0.28 | 11.18±0.59 | 0.10±0.06 | 0.92±0.04 | 4.02±0.57 |
| Lysine | 47.14±0.26 | 9.46±0.37 | 0.06±0.04 | 0.39±0.09 | 7.18±1.12 |
| Isoleucine | 48.65±0.69 | 10.07±0.42 | 0.03±0.01 | 0.26±0.02 | 4.69±1.03 |
| Methionine | 50.65±0.73 | 16.24±0.03 | 0.01±0.00 | 0.02±0.00 | 0.06±0.02 |
| Tyrosine | 48.97±0.72 | 10.41±0.01 | 0.03±0.00 | 0.06±0.00 | 1.92±0.74 |
| Cystine | 46.00±0.11 | 10.76±0.34 | 0.03±0.00 | 0.07±0.02 | 3.93±1.01 |
| L-Hydroxyproline | 44.82±0.83 | 9.01±0.41 | 0.03±0.00 | 0.40±0.07 | 5.03±0.47 |
| Valine | 48.29±0.90 | 9.91±0.14 | 0.03±0.01 | 0.47±0.11 | 5.10±0.11 |
| Histidine | 47.16±0.08 | 9.26±0.11 | 0.03±0.01 | 0.29±0.10 | 6.95±1.01 |
| Arginine | 45.99±0.22 | 10.99±0.53 | 0.03±0.01 | 0.12±0.01 | 4.83±0.08 |
| Tryptophan | 45.51±0.44 | 9.26±0.11 | 0.03±0.00 | 0.25±0.07 | 2.46±0.36 |
| Leucine | 44.93±0.98 | 10.30±0.18 | 0.02±0.00 | 0.46±0.03 | 3.83±0.75 |
| Alanine | 47.27±0.50 | 9.44±0.16 | 0.02±0.00 | 0.43±0.03 | 4.54±0.01 |
| Proline | 47.65±0.53 | 10.11±0.20 | 0.03±0.00 | 0.11±0.00 | 4.73±1.11 |
| Phenylalanine | 43.26±0.02 | 8.46±0.32 | 0.04±0.01 | 0.11±0.04 | 4.57±1.17 |
| Asparagine | 43.99±0.33 | 8.83±0.04 | 0.03±0.01 | 0.29±0.03 | 3.67±0.79 |
| Glycine | 47.94±0.89 | 8.98±0.02 | 0.03±0.00 | 0.41±0.13 | 7.08±0.90 |
| Aspartate | 48.31±0.69 | 9.44±0.15 | 0.03±0.00 | 0.12±0.03 | 2.96±0.38 |
| Glutamine | 48.70±0.70 | 9.13±0.50 | 0.34±0.31 | 0.90±0.13 | 5.77±0.79 |

### 3.5 Selection of Concentration of Amino Acids

Based on the above results, lysine, histidine and glycine were suitable amino acids for further study. The effect of initial lysine, histidine and glycine concentrations on wet weight of medium, dry weight of medium, adenine, adenosine and cordycepin production are given in Table 5. The highest cordycepin production (13.08±0.89 mg/g) was obtained at 6g/L initial histidine. Histidine is therefore a favorable amino acid for production of cordycepin.

**Table 5.** Effects of initial amino acid concentration on the production of adenine, adenosine and cordycepin.

| Amino acid | Concentration (g/L) | Wet weight of medium (g) | Dry weight of medium(g) | Adenine (mg/g) | Adenosine (mg/g) | Cordycepin (mg/g) |
|---|---|---|---|---|---|---|
| Glycine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 49.26±0.73 | 15.63±0.45 | 0.04±0.02 | 0.11±0.04 | 9.18±0.27 |
| | 1 | 49.58±0.71 | 15.08±0.09 | 0.09±0.06 | 0.15±0.02 | 6.92±0.59 |
| | 2 | 51.75±0.76 | 15.38±0.25 | 0.07±0.02 | 0.14±0.04 | 7.76±0.82 |
| | 4 | 49.06±0.74 | 15.64±0.08 | 0.11±0.04 | 0.10±0.03 | 8.43±0.03 |
| | 6 | 46.83±0.39 | 16.27±0.50 | 0.04±0.02 | 0.19±0.03 | 10.37±0.53 |
| | 8 | 48.08±0.01 | 16.22±0.16 | 0.14±0.01 | 0.10±0.04 | 8.05±0.75 |
| | 10 | 48.69±0.88 | 16.57±0.01 | 0.06±0.02 | 0.18±0.03 | 7.71±0.88 |
| Lysine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 49.54±0.28 | 16.75±0.22 | 0.04±0.02 | 0.12±0.03 | 6.70±0.39 |
| | 1 | 48.63±0.09 | 16.45±0.23 | 0.04±0.02 | 0.08±0.02 | 5.62±0.32 |
| | 2 | 48.83±0.80 | 16.75±0.40 | 0.05±0.01 | 0.45±0.03 | 7.58±0.86 |
| | 4 | 50.07±0.52 | 15.84±0.39 | 0.07±0.02 | 0.43±0.01 | 6.55±0.93 |
| | 6 | 51.02±0.51 | 17.26±0.18 | 0.08±0.01 | 0.09±0.03 | 12.69±0.84 |
| | 8 | 49.27±0.06 | 16.43±0.21 | 0.07±0.01 | 0.33±0.09 | 6.14±0.27 |
| | 10 | 51.09±0.04 | 17.28±0.29 | 0.25±0.03 | 0.30±0.08 | 10.92±0.56 |
| Histidine | 0 | 50.69±0.33 | 15.23±0.13 | 0.05±0.00 | 0.07±0.02 | 3.99±0.39 |
| | 0.5 | 48.88±0.81 | 14.82±0.21 | 0.13±0.05 | 0.63±0.05 | 9.30±0.65 |
| | 1 | 49.66±0.36 | 14.62±0.35 | 0.10±0.01 | 0.51±0.06 | 8.45±0.21 |
| | 2 | 49.19±0.99 | 15.67±0.42 | 0.09±001 | 0.55±0.12 | 7.78±0.77 |
| | 4 | 48.40±0.46 | 15.42±0.08 | 0.13±0.00 | 0.56±0.12 | 9.35±0.75 |
| | 6 | 50.42±0.64 | 16.20±0.45 | 0.09±0.02 | 0.54±0.14 | 13.08±0.89 |
| | 8 | 48.49±0.69 | 15.86±0.39 | 0.09±0.03 | 0.56±0.09 | 10.64±0.66 |
| | 10 | 52.32±0.65 | 15.97±0.16 | 0.05±0.03 | 0.34±0.06 | 7.95±0.67 |

### 3.6 Medium Optimization by RSM

In order to evaluate the influence of medium component, concentration of glucose, peptone, adenine and histidine in liquid supplement medium should be examined. The levels of variables for CCD experiments were selected according to the above results of the One-factor-at-a-time method. Figure 2 shows the morphological characteristics of *C. militaris* in 300 mL cylindrical glass bottles at the end of the fermentation process by solid state fermentation. Table 6 shows the detailed experimental design and results. Regression analysis was performed to fit the response function (cordycepin production) with the experimental data. From the variables obtained (Table 7), the model is expressed by Eq.(1), which represents cordycepin production ($Y$) as a function of glucose ($X_1$), peptone ($X_2$), adenine ($X_3$), and histidine ($X_4$) concentrations.

$$Y = 15.28 - 0.84X_1 + 1.04X_2 + 1.79X_3 - 0.71X_4 - 0.96X_2 \times X_3 + 0.93X_2 \times X_4 - 1.53X_3 \times X_4 - 1.26X_3^2 - 0.82X_4^2 \quad (1)$$

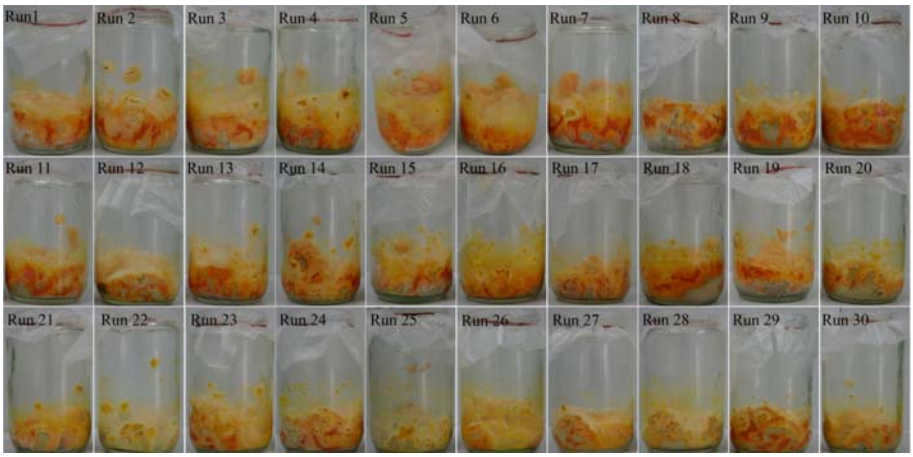


**Figure 2.** Morphology of *C. militaris* CM016 in 300 mL glass jars at the end of the fermentation process by response surface methodology: symbols in photos indicated 30 runs.

**Table 6.** Experimental design and responses of the central composite design (CCD).

| Runs | Independent variables | | | | Dependent variables |
|---|---|---|---|---|---|
| | $X_1$ (g/L) Glucose | $X_2$ (g/L) Peptone | $X_3$ (g/L) Adenine | $X_4$ (g/L) Histidine | $Y$ (mg/g) Cordycepin production |
| 1 | 1 | 1 | 1 | 1 | 10.6586 |
| 2 | 1 | 1 | 1 | 1 | 8.1619 |
| 3 | 1 | 1 | 1 | 1 | 13.2021 |
| 4 | 1 | 1 | 1 | 1 | 12.2889 |
| 5 | 1 | 1 | 1 | 1 | 20.8612 |
| 6 | 1 | 1 | 1 | 1 | 16.8583 |
| 7 | 1 | 1 | 1 | 1 | 17.6851 |
| 8 | 1 | 1 | 1 | 1 | 15.7384 |
| 9 | 1 | 1 | 1 | 1 | 11.3073 |
| 10 | 1 | 1 | 1 | 1 | 8.4972 |
| 11 | 1 | 1 | 1 | 1 | 15.6657 |
| 12 | 1 | 1 | 1 | 1 | 14.9915 |
| 13 | 1 | 1 | 1 | 1 | 13.0189 |
| 14 | 1 | 1 | 1 | 1 | 10.1515 |
| 15 | 1 | 1 | 1 | 1 | 16.2285 |
| 16 | 1 | 1 | 1 | 1 | 13.3909 |
| 17 | 2 | 0 | 0 | 0 | 15.1931 |
| 18 | 2 | 0 | 0 | 0 | 14.385 |
| 19 | 0 | 2 | 0 | 0 | 12.9329 |
| 20 | 0 | 2 | 0 | 0 | 15.5878 |
| 21 | 0 | 0 | 2 | 0 | 5.85646 |
| 22 | 0 | 0 | 2 | 0 | 12.7615 |
| 23 | 0 | 0 | 0 | 2 | 12.2346 |

**Table 6.** Continued.

| Runs | Independent variables | | | | Dependent variables |
|---|---|---|---|---|---|
| | $X_1$ (g/L) Glucose | $X_2$ (g/L) Peptone | $X_3$ (g/L) Adenine | $X_4$ (g/L) Histidine | $Y$ (mg/g) Cordycepin production |
| 24 | 0 | 0 | 0 | 2 | 9.8679 |
| 25 | 0 | 0 | 0 | 0 | 16.1931 |
| 26 | 0 | 0 | 0 | 0 | 14.3834 |
| 27 | 0 | 0 | 0 | 0 | 14.9329 |
| 28 | 0 | 0 | 0 | 0 | 14.5878 |
| 29 | 0 | 0 | 0 | 0 | 15.6783 |
| 30 | 0 | 0 | 0 | 0 | 15.1123 |

**Table 7.** Regression and ANOVA results from the data of central composite design (CCD) experiments.

| Source | Sum of square | df | Mean square | $F$-Value | $p$-value Prob > $F$ |
|---|---|---|---|---|---|
| Model | 256.3 | 9 | 28.48 | 22.09 | < 0.0001** |
| $X_1$ | 16.94 | 1 | 16.94 | 13.14 | 0.0017** |
| $X_2$ | 26.01 | 1 | 26.01 | 20.18 | 0.0002** |
| $X_3$ | 76.93 | 1 | 76.93 | 59.68 | < 0.0001** |
| $X_4$ | 11.95 | 1 | 11.95 | 9.27 | 0.0064** |
| $X_2X_3$ | 14.77 | 1 | 14.77 | 11.45 | 0.0029** |
| $X_2X_4$ | 13.93 | 1 | 13.93 | 10.8 | 0.0037** |
| $X_3X_4$ | 37.53 | 1 | 37.53 | 29.11 | < 0.0001** |
| $X_3^2$ | 44.89 | 1 | 44.89 | 34.82 | < 0.0001** |
| $X_4^2$ | 19.16 | 1 | 19.16 | 14.86 | 0.001** |
| Residual | 25.78 | 20 | 1.29 | | |
| Lack of Fit | 23.46 | 15 | 1.56 | 3.37 | 0.0927 |
| Pure Error | 2.32 | 5 | 0.46 | | |
| Cor Total | 282.09 | 29 | | | |

$R^2$ = 0.9146; CV = 8.34%; Pred-$R^2$ = 0.7562; Adj-$R^2$ = 0.8675; Adeq Precision = 18.686; ** 1% significance level.

Results of $F$-test analysis of variance (ANOVA) showed that the regression was statistically significant ($P < 0.05$) at the 95% confidence level (Table 7). The model presents a higher regression coefficient ($R^2$= 0.9086).

The response surface plot obtained from Eq. (1) is shown in Figure 3. It is evident that cordycepin production reached its maximum at a combination of coded level ($X_1$, glucose, level 1; $X_2$, peptone, level 1; $X_3$, adenine, level 1; $X_4$, histidine level 1) by canonical analysis of the Design-expert Version 8.0.5b software package. The model predicted a maximum response of 18.92 mg/g cordycepin production at levels of glucose 26.25 g/L, peptone 26.25 g/L, adenine 7.50 g/L, histidine 4.50 g/L as optimized medium components.

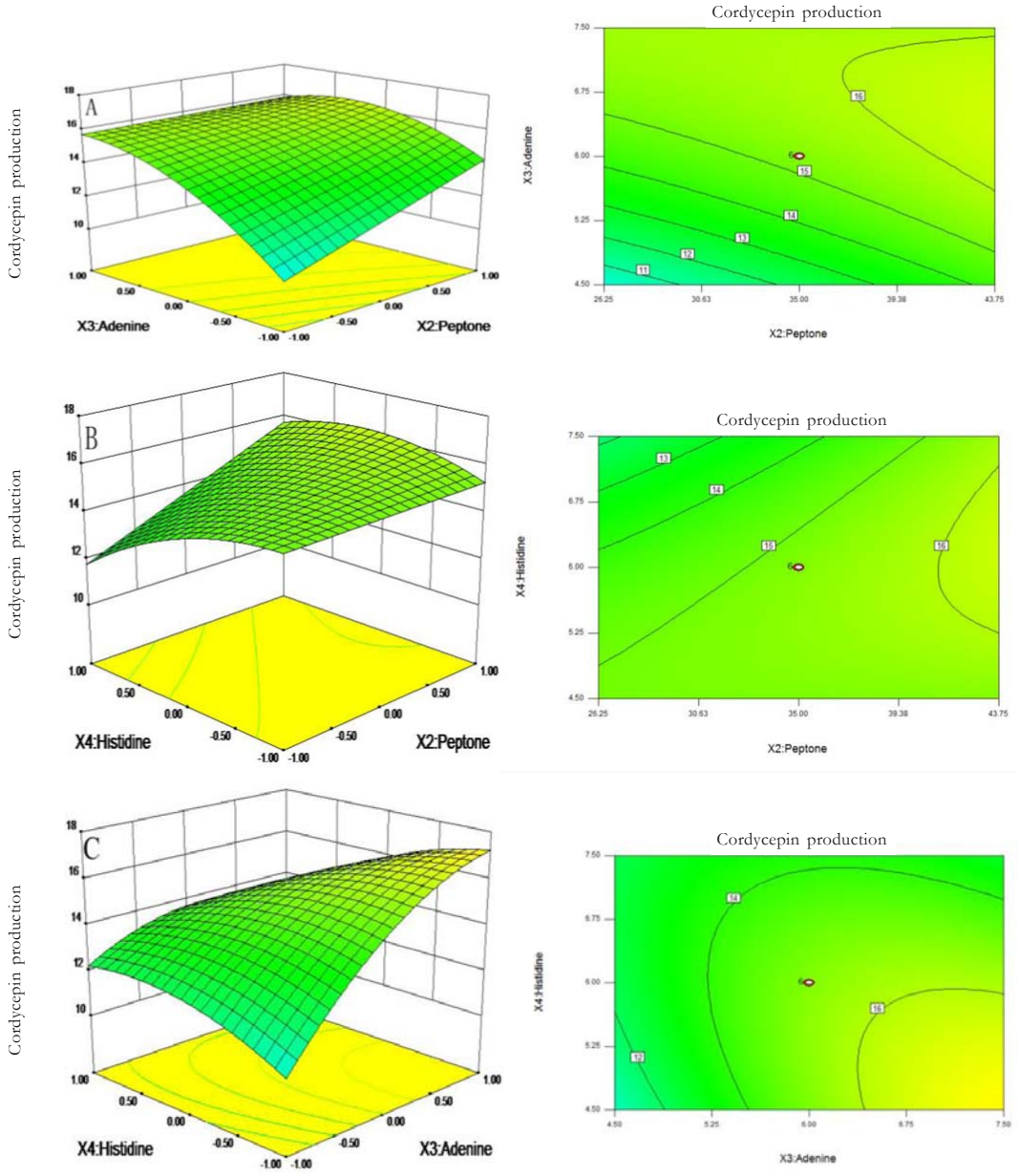


**Figure 3**. Three-dimensional response surface plots and two-dimensional contour plots for cordycepin production by *C. militaris* CM016 showing variable interactions of: (A) peptone and adenine; (B) peptone and histidine; (C) adenine and histidine.

### 3.7 Cordycepin Production in Fruit Body and Medium by Solid-state Fermentation in Different Studies

In this paper, we compare the differences of fruit body weight, cordycepin production of fruit bodies, cordycepin production in medium, or incubation time by solid state fermentation in different studies. As shown in table 8, the results were not consistent in different studies. The highest report for fruit body weight was 12.9 g/bottle at 60 days [17], and the maximum cordycepin

production in fruit bodies was 18.7 mg/g at 70 days by Wen *et al.* [18]. They needed long incubation periods in these studies. In this study, the cordycepin production (18.92 mg/g) was obtained in medium at 39 days. This is the highest report of cordycepin production in residue medium by solid-state fermentation. The results show that the optimized culture conditions will provide an effective way for increasing cordycepin production.

**Table 8.** Fruit body weight, cordycepin production in fruit body and medium by solid-state fermentation in different studies.

| No. | Strain | Fruit body weight (g) | Cordycepin in fruit body (mg/g) | Cordycepin in medium (mg/g) | Cultivation time (days) | Reference |
|---|---|---|---|---|---|---|
| 1 | *C. militaris* CM-2 | - | - | 5.49 | 35 | Wen *et al.*, [24] |
| 2 | *C. militaris* | 8.14 | -[+] | - | 50 | Sung *et al.*, [25] |
| 3 | *C. militaris* | - | 9.7 | - | | Hur, [4] |
| 4 | *C. militaris* JF-1 | - | - | 6.0 | 13 | Wei *et al.*, [26] |
| 5 | *C. militaris* 067 | 1.35 | 18.7 | 2.1 | 70 | Wen *et al*, [18] |
| 6 | *C. militaris* cmily-02 | - | - | 1.85 | 16 | Chen *et al.*, [27] |
| 7 | *C. militaris* | - | 4.6 | - | 57-60 | Xiao *et al*, [28] |
| 8 | *C. cardinalis* C-10376 | 12.9 | - | - | 60 | Kim *et al.*, [17] |
| 9 | *C. militaris* | - | 5.57 | - | 50 | Dong *et al.*, [29] |
| 10 | *C. militaris* CM016 | - | - | 18.92 | 39 | In this study |

[+]: Data deficient

## 4. DISCUSSIONS

Metabolite production by microorganism is a dynamic process which changes with cultivation times and culture conditions**.** Cordycepin (3′-deoxyadenosine), was first isolated from *C. militaris* [5], and is one of the most important biologically active metabolites found in this species. Fruiting bodies of *C. militaris* take long cultivation periods to develop and is difficult to achieve large-scale production via solid-state fermentation [18]. Cordycepin is also low in the fruiting bodies [4]. About 98% of the cordycepin synthesized by *C. militaris* was secreted into the culture medium in previous studies [11]. In this study, cordycepin production was studied by solid-state fermentation by *C. militaris* CM016. The results show that a maximal cordycepin production of 3.3 ± 0.03 mg/g occurred on day 39 (Figure. 1). However, cordycepin production reduced gradually with increasing cultivation time from 39 to 65 days.

In order to improve production of the metabolites, precursors and/or additives are added to the fermentation process. Amino acids and nucleoside are better precursors and/or additives in liquid fermentation of *C. militaris* [19]. In this study, amino acids and nucleoside were added to the basal medium by solid state culture, respectively. As a result, most of amino acids and nucleosides can increase the yield of cordycepin production, and lysine, histidine, glycine, adenine, cytosine and thymidine were shown to be effective additives. The result is similar to that of other reports

[19]. Initial nucleoside and amino acids concentrations were screened. The results show that the best nucleoside and amino acid were adenine and histidine, and the optimum concentrations were 6 g/L and 6 g/L in the basal liquid medium respectively.

Response surface methodology (RSM) was used to optimize the screened variables for enhanced metabolite production [20]. In previous work, the orthogonal design method [21], Box-Behnken design [22], and central composite design [15] were used to optimize culture conditions for cordycepin production by liquid fermentation in *Cordyceps* sp. In this study, solid state fermentation conditions are optimized for the cordycepin production using the response surface methodology.

In order to evaluate the influence of medium component on cordycepin production in rice medium, glucose, peptone, adenine, and histidine should be examined. The model predicted a maximum production of 18.92 mg/g cordycepin at 26.25 g/L glucose, 26.25 g/L peptone, 7.50 g/L adenine, and 4.50 g/L histidine as optimized medium components. In previous work, cordycepin production in liquid medium has been significantly enhanced through adding adenine and glycine [19, 23], or hypoxanthine and L-alanine [15]. This is the first report for improved the cordycepin content in rice medium using solid-state fermentation. Adenine and histidine were better additives for cordycepin production in this study. The reason may be the complex component of solid medium, and this is a case for subsequent study. As shown in table 7, this is the highest report of cordycepin production in medium by solid state fermentation. Substrates source for solid state fermentation are abundant, the cost is low, and the physical conditions are not costly.

## 5. CONCLUSIONS

In this work, the one-factor-at-a-time method and central composite design were employed to establish the key factors and identify optimal culture conditions to improve cordycepin production by *C. militaris* in solid state fermentation. Optimal medium contained glucose 26.25 g/L, peptone 26.25 g/L, adenine 7.50 g/L, histidine 4.50 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L and $KH_2PO_4$ 0.1 g/L in liquid supplement medium. The optimal medium for solid state fermentation was prepared by mixing 20 g of rice and 32 mL of optimal liquid supplement medium in 300 mL cylindrical glass bottle. Using these culture conditions, a maximum production of cordycepin was 18.92 mg/g. This method provides an effective way for increasing cordycepin production by solid state fermentation.

## ACKNOWLEDGMENTS

This work was supported by the Agricultural Science and Technology Foundation of Guizhou Province (No. [2011]3054), the National Natural Science Foundation of China (No. 31200016), the Modernization of Traditional Chinese Medicine Program of Guizhou Province (No. [2012]5008), and the Youth Foundation of Guizhou Academy of Sciences (No. [2014]07).

## REFERENCES

[1] De Silva D.D., Rapior S., Sudarman E., Stadler M., Xu J., Alias S.A. and Hyde K.D., *Fungal Divers.*, 2013; **62(1)**: 1-40. DOI 10.1007/s13225-013-0265-2.

[2] Xu J.T., *Medicinal Fungus in China (Chinese)*, Beijing Medical University, Peking Union Medical College Joint Press, Beijing, China, 1997.

[3] Mortimer P.E., Karunarathna S.C., Li Q., Gui H., Yang X., Yang X., He J., Ye L.,

Guo J. and Li H., *Fungal Divers.*, 2012; **56(1)**: 31-47. DOI 10.1007/s13225-012-0196-3.

[4] Hur H., *Mycobiology*, 2008; **36(4)**: 233-235. DOI 10.4489/MYCO.2008.36.4.233.

[5] Cunningham K.G., Manson W. and Spring F.S., *Nature*, 1950; **166(4231)**: 949. DOI 10.1038/166949a0.

[6] De Silva D.D., Rapior S., Fons F., Bahkali A.H. and Hyde K.D., *Fungal Divers.*, 2012; **55(1)**: 1-35. DOI 10.1007/s13225-012-0151-3.

[7] Sugar A.M. and McCaffrey R.P., *Antimicrob. Agents Ch.*, 1998; **42(6)**: 1424-1427.

[8] Guo P., Kai Q., Gao J., Lian Z., Wu C., Wu C. and Zhu H., *J. Pharmacol. Sci.*, 2010; **113(4)**: 395-403. DOI 10.1254/jphs.10041FP.

[9] Wen T.C., Li M.F., Kang J.C. and He J., *Afr. J. Microbiol. Res.*, 2012; **6(24)**: 5215-5221. DOI 10.5897/AJMR12.522.

[10] Shrestha B., Han S., Sung J. and Sung G., *Mycobiology*, 2012; **40(2)**: 100-106. DOI 10.5941/MYCO.2012.40.2.100.

[11] Masuda M., Urabe E., Sakurai A. and Sakakibara M., *Enz. Microb. Technol.*, 2006; **39(4)**: 641-646. DOI 10.1016/j.enzmictec.2005.11.010.

[12] Wen T.C., Kang J.C., Hyde K.D., Li G.R., Kang C. and Chen X., *Chiang Mai J. Sci.*, 2014; **4(41)**: 846-857.

[13] Wen T.C., Kang J.C., Lei B.X., Li G.R. and He J., *Food Sci. (in Chinese)*, 2010; **31(5)**: 175-179.

[14] Kang C., Wen T.C., Kang J.C., Qian Y.X. and Lei B.X., *Mycosystem (in Chinese)*, 2012; **31(3)**: 389-397.

[15] Kang C., Wen T.C., Kang J.C., Meng Z.B., Li G.R. and Hyde K.D., *The Scientific World J.*, 2014; DOI 10.1155/2014/510627.

[16] Wen T.C., Kang J.C., Lei B.X., Li G.R. and He J., *Guizhou Agric. Sci. (in Chinese)*, 2008; **36(4)**: 92-94.

[17] Kim S.Y., Shrestha B., Sung G.H., Han S.K. and Sung J.M., *Mycobiology*, 2010; **38(2)**: 133-136. DOI 10.4489/MYCO.2010.38.2.133.

[18] Wen T.C., Kang J.C., Lei B.X., Li G.R. and He J., *Guizhou Agric. Sci. (in Chinese)*, 2008; **4(36)**: 92-94.

[19] Masuda M., Urabe E., Honda H., Sakurai A. and Sakakibara M., *Enz. Microb. Technol.*, 2007; **40(5)**: 1199-1205. DOI 10.1016/j.enzmictec.2006.09.008.

[20] Guo W., Ren N., Wang X., Xiang W., Ding J., You Y. and Liu B., *Bioresour. Technol.*, 2009; **100(3)**: 1192-1196. DOI 10.1016/j.biortech.2008.07.070.

[21] Xiao J.H., Chen D.X., Xiao Y., Liu J.W., Liu Z.L., Wan W.H., Fang N., Tan B.B., Liang Z.Q. and Liu A.Y., *Process Biochem.*, 2004; **39(12)**: 2241-2247. DOI 10.1016/j.procbio.2003.11.026.

[22] Shih I., Tsai K. and Hsieh C., *Biochem. Eng. J.*, 2007; **33(3)**: 193-201. DOI 10.1016/j.bej.2006.10.019.

[23] Das S.K., Masuda M., Sakurai A. and Sakakibara M., *Afr. J. Biotechnol.*, 2009; **8(13)**: 3041-3047. DOI 10.4314/ajb.v8i13.60983.

[24] Wen L., Xia M., Song H.W., Zhang L., Zhou H., Jiang J. and Yuan C.S., *Food Sci.*, 2005; **26(11)**: 65-68.

[25] Sung J., Park Y., Lee J., Han S., Lee W., Choi S. and Shrestha B., *Mycobiology*, 2006; **34(3)**: 131-137. DOI 10.4489/MYCO.2006.34.3.131.

[26] Wei H.P., Ye X.L., Zhang H.Y., Li X.G. and Zhong Y.J., *China J. Chin. Materia Medica (in Chinese)*, 2008; **19(33)**: 2159-2162.

[27] Chen Z.H., Yu H., Zeng W.B., Yang J.Y., Yang Z.L. and Yuan J., *Edible Fungi of China (in Chinese)*, 2009; **6(28)**: 45-47.

[28] Xiao Z.H., Li Z.X., Li J.Z., Zou L.K. and Wang T., *Food Ferment. Technol. (in Chinese)*, 2010; **3(46)**: 60-64.

[29] Dong J.Z., Lei C., Ai X.R. and Wang Y., *Appl. Biochem. Biotechnol.*, 2012; **166(5)**: 1215-1224. DOI 10.1007/s12010-011-9506-6.