Process Biochemistry 45 (2010) 129–132

Contents lists available at ScienceDirect

Process Biochemistry

journal homepage: www.elsevier.com/locate/procbio

Short communication

Optimization of culture medium for cordycepin production using *Cordyceps militaris* mutant obtained by ion beam irradiation

Shonkor Kumar Das [a], Mina Masuda [a], Masanori Hatashita [b], Akihiko Sakurai [a], Mikio Sakakibara [a,*]

[a] Department of Applied Chemistry and Biotechnology, Graduate School of Engineering, University of Fukui, Fukui 910-8507, Japan
[b] Research & Development Department, The Wakasa-wan Energy Research Center, Tsuruga 914-0192, Japan

ARTICLE INFO

Article history:
Received 22 May 2009
Received in revised form 29 July 2009
Accepted 10 August 2009

Keywords:
Cordyceps militaris mutant
Cordycepin
Surface liquid culture
Medium optimization
Response surface analysis
Central composite design

ABSTRACT

The effect of medium components on cordycepin production by *Cordyceps militaris* mutant obtained by ion beam irradiation was investigated. According to the response surface analysis using a central composite design for the prospective mutant G81-3, the predicted optimal concentrations of glucose as the carbon source and the yeast extract as the nitrogen source were 86.2 g/l and 93.8 g/l, respectively, and 6.84 g/l cordycepin was obtained. To date, this is the highest value for cordycepin production. The optimal concentrations of glucose and yeast extract for cordycepin production of the mutant was much higher than that of control (wild strain) and the cordycepin production was 2.79 times higher. Therefore, this new mutant will be a promising strain for future higher cordycepin production at industrial levels.

1. Introduction

The medicinal mushrooms are abundant sources of useful natural products with various biological activities [1]. Therefore, many researchers in the field of physiology, pharmacology and medical science, etc., are interested in the contents of mushrooms.

Among various mushrooms, *Cordyceps* spp. have been extensively used as the crude drug or folk tonic food in East Asia. The *Cordyceps* genus is a name given to the medicinal mushroom that parasitizes in insects and changes itself to fruiting bodies, and then comes out of the ground. As it appears like a plant growing out of an insect, it is given the self-explanatory name Tochukaso (winter insect–summer plant) in Japan. One of the most important species in *Cordyceps* genus is *Cordyceps militaris* (an entomopathogenic fungus), which belongs to the class *Ascomycetes*. *C. militaris* contains many kinds of active components, such as cordycepin (3′-deoxyadenosine), ergosterol, mannitol and polysaccharides, and due to its various physiological activities, is now used for multiple medicinal purposes [2,3].

The cordycepin biosynthesized in *C. militaris* is a unique nucleoside analogue, which has a broad spectrum of biological activity, especially as an anti-cancer drug [4,5]. In nature, *C. militaris* is very scarce due to the requirements of specific hosts and strict growth environments. Therefore, a large-scale production of cordycepin by the cultivation of *C. militaris* is currently a significant issue.

To obtain a good productivity, a good strain and an optimized culture condition for it are necessary. As for the good producer of cordycepin, we previously reported a new approach for obtaining a novel mutant of *C. militaris* using high-energy ion beam irradiation [6]. The obtained mutant, G81-3, had 72% higher cordycepin production than the wild strain in preliminary optimization. As for the culture condition, medium composition, especially carbon and nitrogen sources, is important in the yield of any fermentation products [7–10,11]. However, 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 using surface liquid culture. Therefore, in this study, the effect of medium components on mutant strain was analyzed using the response surface method and the medium components for the cordycepin production was optimized.

2. Materials and methods

2.1. Fungal strain, media and culture

C. militaris NBRC 9787 and a prospective mutant (G81-3) of the said strain obtained by ion beam irradiation [6] were used for experiments. The surface liquid culture for *C. militaris* mutant and control (wild strain) were carried out as described in our previous research [6]. All experiments were carried out at least in duplicate, and the results were averaged.

2.2. Optimization of medium composition using central composite design (CCD)

In order to explore the effect of the medium components (glucose and yeast extract) and to optimize them, a statistical approach using the Box–Wilson central

* Corresponding author. Tel.: +81 776 27 8634; fax: +81 776 27 8747.
E-mail address: m-sakaki@u-fukui.ac.jp (M. Sakakibara).

1359-5113/$ – see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.procbio.2009.08.008

composite design (CCD) was conducted [12]. The variable levels for the CCD experiments were set according to the results of our previous experiments. The CCD experimental results were fitted with a second-order polynomial equation of Eq. (1) using a multiple regression technique:

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

where y is the predicted response (peak value of cordycepin concentration), β_0 is a constant coefficient, β_i is the linear coefficient, β_{ij} is the interaction coefficient, β_{ii} is the quadratic coefficient, and x_i and x_j are, respectively, the actual values of glucose and yeast extract concentrations.

In this study, the experimental design consisted of 13 trials (1–8: star and factorial points, each has two replications; 9–13: center points), and the independent variables were studied at five different levels; as −1.414, −1, 0, +1 and +1.414. The analysis of variance (ANOVA) was performed to evaluate significance of the model and coefficients. This analysis included the Fisher's F-test (overall model significance) with the associated probability p-value. All the above analyses were done using "Design Expert" software with the version 7.0 (Stat-Ease Inc., USA) statistical package.

Finally, the surface liquid culture with seven replications was conducted for the validation of the predicted values (cordycepin productions using optimized medium) for both the mutant and wild strain, and then the results were averaged.

2.3. Analytical procedures

The cordycepin concentration was determined by an HPLC (LC-9A system, Shimadzu Corp., Japan) under the following conditions: TSK-gel ODS-80Ts (Tosoh Corp., Japan); mobile phase, methanol and 0.1% phosphoric acid (2/98, v/v); flow rate, 1.0 ml/min; column temperature, 40 °C and peak detection, UV at 260 nm. The cordycepin concentration shown in this experiment was re-estimated by considering the condensation of the medium in the culture bottle due to vaporization.

The glucose concentration was analyzed by the mutarotase–glucose oxidase method using the Glucose CII test Wako (Wako Pure Chemical Industries, Ltd., Japan). For the measurement of dry mycelia weight, at the end of each culture (glucose concentration < 0.5% of the initial concentration), the entire remaining content in the bottle was centrifuged at 16,000 × g and 4 °C for 20 min. The precipitated mycelia were then sufficiently washed with distilled water, and dried at 105 °C for 24 h to measure the dry mycelia weight.

3. Results and discussion

3.1. Optimization of cordycepin production by response surface methodology

The combined effect of the carbon source (glucose) and nitrogen source (yeast extract) was investigated using CCD, which can help to identify the interaction between the variables. The trace element except C and N sources were the same as the previous experiments [6,13,14]. In our preliminary experiments using CCD, that is, the center point was 40 g/l of glucose and 35 g/l of yeast extract and the intervals were 10 g/l for both the mutant and the wild strain, the cordycepin productions by these strains increased as the glucose and yeast extract concentrations increased. The optimal point for the mutant was predicted in higher region than that for the wild strain (data not shown). These results could not contradict with the previous report showing that the metabolic ability of the mutant was higher than that of the wild strain [6].

Based on the above mentioned result, the CCD experiment shown in Table 1(a-1) was first conducted for the mutant, and the predicted concentrations were higher than the experimental range. Then, the experimental ranges were shifted to high concentrations as shown in Table 1(a-3). The results showed that there was no mycelial growth in some culture bottles having a high glucose or yeast extract content. Finally, the CCD experiment shown in Table 1(a-2) was conducted to complement the range between Table 1(a-1) and (a-3). All the three experiments were merged to make a single response surface plot in order to detect the optimized medium concentrations along with the prediction of maximum cordycepin production.

For the wild strain, the predicted medium conditions and cordycepin production were obtained from the experiment having a center point concentration of 60 g/l for both glucose and yeast extract, with the ranges from 31.72 g/l to 88.28 g/l as shown in Table 1(b).

Table 1
Coded and actual values of design factors for investigation of medium components for the (a) mutant and (b) wild strain.

Coded levels	Actual factor levels	
	Glucose (x_1)	Yeast extract (x_2)
(a-1)		
−1.414	35.86	60.86
−1	40	65
0	50	75
1	60	85
1.414	64.14	89.14
(a-2)		
−1.414	36.72	56.72
−1	45	65
0	65	85
1	85	105
1.414	93.28	113.28
(a-3)		
−1.414	71.72	91.72
−1	80	100
0	100	120
1	120	140
1.414	128.28	148.28
(b)		
−1.414	31.72	31.72
−1	40	40
0	60	60
1	80	80
1.414	88.28	88.28

A regression analysis was performed to fit the response (cordycepin production) with the experimental data. Based on the obtained variables, the responses were, respectively, expressed by Eqs. (2) and (3) for the mutant and the wild strain, which presented the cordycepin production y (g/l) as a function of the glucose x_1 (g/l) and yeast extract x_2 (g/l) concentrations.

$$y = -10.14958 + 0.17365x_1 + 0.20110x_2 - 0.00101x_1^2 - 0.00107x_2^2 \text{ (mutant)} \quad (2)$$

$$y = -1.37420 + 0.06007x_1 + 0.05443x_2 - 0.00048x_1^2 - 0.00038x_2^2 \text{ (wild strain)} \quad (3)$$

In both cases, there are no interactions between glucose and yeast extract concentrations. Results of the analysis of variance (ANOVA) showed that the regression model was statistically significant at a 95% confidence level ($p < 0.05$) (Table 2; where x is

Table 2
Results of ANOVA for optimization of medium components [(a) mutant and (b) wild strain].

Source	Sum of squares	df	F-value	p-value	Significance
(a)					
Model	36.97	5	9.99	<0.0001	Significant
x_1: Glucose	24.49	1	33.09	<0.0001	Significant
x_2: Yeast extract	4.49	1	6.06	0.0184	Significant
x_1x_2	1.06	1	1.43	0.2387	
x_1^2	10.19	1	13.77	0.0007	Significant
x_2^2	5.73	1	7.75	0.0083	Significant
(b)					
Model	0.91	5	9.08	<0.0007	Significant
x_1: Glucose	0.039	1	1.94	0.1874	
x_2: Yeast extract	0.56	1	28.05	0.0001	Significant
x_1x_2	0.000032	1	0.001592	0.9688	
x_1^2	0.28	1	14.08	0.0024	Significant
x_2^2	0.17	1	8.61	0.0116	Significant

Significant: $p < 0.05$.