



Improvement of cordycepin production by an isolated *Paecilomyces hepiali* mutant from combinatorial mutation breeding and medium screening

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Abstract

Cordycepin is a major bioactive compound found in *Cordyceps sinensis* that exhibits a broad spectrum of biological activities. Here a *Paecilomyces hepiali* OR-1 strain was initially isolated from plateau soil for the bioproduction of cordycepin. Subsequently, strain modification including ⁶⁰Co γ -ray and ultraviolet irradiation were employed to increase the cordycepin titer, resulted in a high-yield mutant strain *P. hepiali* ZJB18001 with the cordycepin content of 0.61 mg/g_{DCW}, showing a 2.3-fold to that from the wild strain (0.26 mg/g_{DCW}). Furthermore, medium screening based on Box-Behnken design and the response surface methodology facilitated the enhancement of cordycepin yield to the value of 0.96 mg/g_{DCW} at 25 °C for 5 days in submerged cultivation with an optimized medium composition. The high cordycepin yield, rapid growth rate and stable genetic characteristics of *P. hepiali* ZJB18001 are beneficial in terms of costs and time for the industrialization of cordycepin production.

Keywords Cordycepin · ⁶⁰Co- γ ray irradiation · *Paecilomyces hepiali* · Medium screening · Response surface methodology

Introduction

Cordycepin is a derivative of the nucleoside adenosine with the hydroxy group absent at the 3' position of its ribose moiety (3'-deoxyadenosine, C₁₀H₁₀N₅O₃, Fig. 1) [1]. As the first antibiotic nucleoside isolated from *Cordyceps* genus, cordycepin is well-known for a variety of biological and pharmacological activities [2, 3]. Applications in cosmeceutical as a bioactive ingredient are expanding as well as anti-photoaging and anti-pigmentation [4]. However, the market price of cordycepin keeps high over the years due to the depletion and slow growth of the wild resources of *Cordyceps sinensis* caused by predatory excavation and

environmental destruction. How to realize the cordycepin production in an industrial scale has therefore attracted the interests of researchers especially in Asia.

Biological production of cordycepin could be achieved by direct extraction from *C. militaris* fruiting body, or from its fermentation broth through solid-state or liquid fermentation [5, 6]. It takes 60 days for the *C. militaris* fruiting body to be ready for extraction and the culture conditions are complex and hard to control as well. In comparison, the production cycle of liquid fermentation is shorter (~ 15 days), and the fermentation process is easier to control [2, 7]. Liquid fermentation technology has therefore become the major means of cordycepin production with a bunch of work in cordycepin-producing fungi such as *Cordyceps militaris*, *Cordyceps sinensis*, *Cordyceps kyushuensis*, *Aspergillus nidulan*, and *Paecilomyces hepiali*, which exhibited high similarity in components and efficacy of active compounds [2, 4, 7, 8].

The low cordycepin productivity has been improved by strategies including physical and chemical mutagenesis breeding, process optimization, genetic and metabolic engineering [9]. Among them, mutagenesis breeding technologies such as ultraviolet, γ -ray irradiation, plasma, and chemical mutagens have been considered as practical

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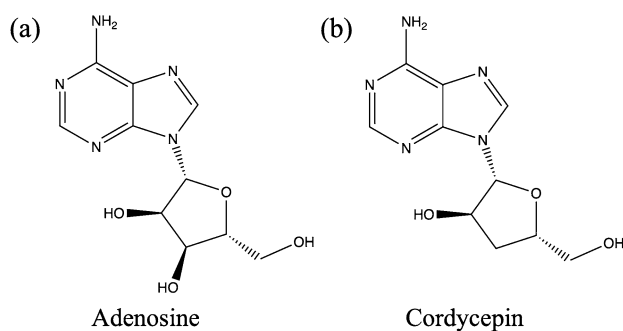


Fig. 1 Chemical structure of adenosine (a) and cordycepin (b)

approaches in the industry for decades [10]. γ -Rays produced by the radioisotope ^{60}Co or ^{137}Cs are high-energy electromagnetic waves which are able to penetrate through the cell walls of mycelia, which would cause DNA sequence mutation. Liu et al. [11] reported a successful example of ^{60}Co γ -irradiation on an edible mushroom *Agaricus brasiliensis*, resulting in a mutant with 50% lower Cd accumulation and 40.3% higher biological efficiency than those of the parent. As far as cordycepin production is concerned, proton beam irradiation has been employed on *Cordyceps militaris* with a cordycepin titer increased to 14.3 g/L [12–14]. *Cordyceps militaris* KYL05 was also mutated by UV irradiation with a 2.3-fold improvement in cordycepin production [15]. An extensive literature search showed that there has not yet any study reporting using ^{60}Co γ -ray irradiation for cordycepin production improvement.

Medium optimization along with the response surface methodology (RSM) is another effective approach to enhance the yield and to determine key factors influencing the process with limited experiments as it is a combination of mathematical and statistical techniques [16, 17]. A recent research has reported additional growth supplements such as nucleosides-hypoxanthine increased the cordycepin titer by 34-fold in mycelial biomass of *Ophiocordyceps sinensis* [18]. Tang et al. [19] evaluated different vegetable oils as the second carbon source of *C. militaris* resulting in 3.17 times higher cordycepin titer than control using 20 g/L peanut oil. Casein hydrolysate was also found effective as a nitrogen source with a 2.3-fold improvement to 445 mg/L [15].

The present work isolated and identified a *P. hepiali* mutant strain from the mutation library of the wild *C. sinensis* by ^{60}Co γ -ray and ultraviolet irradiation. Further attempt was made to optimize the basic culture medium for achieving a high level of cordycepin by the mutant strain using Box-Behnken design and response surface methodology in a four-factor three levels experiment.

Materials and methods

Chemical reagents

Standard cordycepin was purchased from Sigma-Aldrich (Shanghai, China). Methanol of HPLC grade was purchased from Honeywell Burdick & Jackson Chemical Ltd. (New Jersey, USA). All other chemical reagents were of analytical grade and commercially available.

Microorganism isolation and cultivation

Wild *Cordyceps sinensis* samples were collected from Qinghai, China, washed with tap water (3 times), distilled water (3 times) and 0.1% mercuric chloride solution for surface disinfection, sucked dry with sterile paper on a clean bench. The body parts were dissected with a sterile scalpel and transplanted onto potato dextrose agar plate (PDA plate) containing 80 mg/L of gentamicin. The plates were incubated at 10 °C for everyday observation until white mycelia without contamination were selected for transplantation and cultivated on new PDA plates without gentamicin at 25 °C for screening. A single colony was inoculated into 50 mL of seed medium (glucose 20 g/L, peptone 5 g/L, yeast extract 2 g/L, KH_2PO_4 1 g/L and MgSO_4 0.5 g/L) in a 250 mL flask, and incubated at 25 °C for 3 days. The fermentation medium contained glucose 20 g/L, peptone 5 g/L, KH_2PO_4 1 g/L and MgSO_4 0.5 g/L. Unless otherwise specified, fermentations were carried out in a 250 mL flask at 25 °C and an initial pH of 6.0 for 5 days, with 2% (v/v) inoculation of the seed culture. The morphological observations were carried out by an optical microscope (DM3000, Leica, Germany) and an environmental scanning electron microscope (ESEM, SU-8010, Hitachi, Tokyo, Japan) with the samples treated as Abrusci et al. [20] reported for SEM observation.

The wild strain was cultivated in a 5 L bioreactor (Shanghai Baoxing Biological Equipment Engineering Co. Ltd, China) with 150 mL inoculation and a working volume of 3 L using optimized fermentation media. Cells were incubated at 25 °C for 5 days with agitation at 150 rpm and 6 L/min filter sterilized airflow with automatic pH adjustment by ammonia to keep the pH at 6.0 and foaming control by sterile polypropylene glycol addition. Samples were taken every 12 h to measure the dry cell weight and cordycepin titer.

Microorganism identification by 18S rDNA and Biolog system

The 18S rDNA of the newly isolated microorganism was extracted and enzymatically amplified according to a previously described method [21], sequenced and compared in the

NCBI database by using the Basic Local Alignment Search Tool (BLAST, <http://www.ncbi.nlm.nih.gov/BLAST>). The neighbor-joining tree was constructed using the Clustal Omega program. The utilizations of carbon and nitrogen sources of the isolated microorganism were studied with Biolog system kit following the manual (Biolog, Hayward, CA, USA).

Combinatorial mutation breeding of ⁶⁰Co γ-ray and ultraviolet irradiation

The suspension of strain *P. hepiali* OR-1 with 1.0 × 10⁶ cells/mL was prepared with 0.1% of Tween80 for ⁶⁰Co γ-ray and ultraviolet irradiation sequentially. The radiation process was conducted in the Irradiation Center of Zhejiang Academy of Agricultural Sciences (Hangzhou, China) using the HFY-YC type γ-ray radiation device with radiation doses of 100, 200, 400, 600, 800 and 1000 Gy. Eight samples were irradiated for each dose at the same time. The suspension was placed in ice bath until use after irradiation. The retrieved spore solution was diluted to reach an even spore concentration of 1.0 × 10³ spore/mL and 50 μL of the diluted spore solution was added onto the PDA plate at 25 °C for 3 days. Colony counts were then performed to determine the death rates. Single colonies were picked for cultivation and cordycepin content determination.

The mutant strain with the highest cordycepin content obtained by ⁶⁰Co γ-ray irradiation was stored and prepared in 10⁶ spores/mL as a suspension solution, evenly placed onto a sterile plate to form a 2 mm-thick film for ultraviolet irradiation with a 15 W UV lamp (emission wavelength of 253.7 nm). The lengths of ultraviolet irradiation were of 5 min, 10 min, 15 min, 20 min, 25 min and 30 min, respectively, and immediately placed on ice for 1 h in dark. A volume of 100 μL diluted samples (100 spore/mL) were coated on PDA plates in triplicate and incubated at 25 °C for 3 days in the dark.

The lethal rate, positive and negative mutation rates of ⁶⁰Co γ-ray and ultraviolet irradiation were calculated according to Eqs. (1), (2) and (3), respectively. The positive/negative mutation rate was defined as the cordycepin yield increased/decreased by over 10% comparing to the parent. Equations (1), (2) and (3) were as follows:

$$\text{Lethal rate(\%)} = \frac{N_0 - N_1}{N_0} \times 100\% \quad (1)$$

$$\text{Positive mutation rate(\%)} = \frac{N_{\Delta\text{Cordycepin}>+10\%}}{N_1} \times 100\% \quad (2)$$

$$\text{Negative mutation rate(\%)} = \frac{N_{\Delta\text{Cordycepin}<-10\%}}{N_1} \times 100\% \quad (3)$$

*N*₀ indicates the number of viable bacteria before irradiation, *N*₁ indicates the number of viable bacteria after irradiation.

Study on the genetic stability of the mutant strain

The genetic stability of the obtained mutant strain *P. hepiali* ZJB18001 was tested by cultivation as the standard cultivation procedure as described in “Microorganism identification by 18S rDNA and Biolog system” for 10 generations. Samples from each generation were taken for cordycepin quantification by HPLC.

Medium screening

Single factor optimization

Nine carbon sources of glucose, D-fructose, sucrose, maltose, mannitol, corn syrup, molasses, peanut oil, methyl oleate in a concentration of 20 g/L and eight nitrogen sources of peptone, yeast extract, beef extract, silkworm chrysalis, peanut powder, yeast extract, bran, soybean meal of 10 g/L were tested, respectively. Besides, metal ions including Ca²⁺, Mn²⁺, Zn²⁺, Fe²⁺, Cu²⁺, Al³⁺ and Fe³⁺ with sulfate ions of 4 mmol/L and precursors including adenosine, adenine, guanosine, guanine, uridine, uracil, thymidine, inosine and D-ribose of 2 g/L were added separately to further optimize the fermentation medium.

Response surface methodology

Four factors (peanut oil, yeast extract, metal ion Fe³⁺ and precursor adenosine) at three different levels (− 1, 0, 1) were following the Box-Behnken design (Table 1). A set of 29 experiments was generated with five replicates at the center point for enhanced cordycepin production. A second-order polynomial model was used to fit the responses to the values of the factors.

Validation of the model

Experiment was performed in triplicate, to verify the statistical model at the optimal values of four factors. The observed

Table 1 Concentration ranges of the four factors used in the central composite design

Factor	Actual levels of coded factors		
	− 1	0	+ 1
Peanut oil (g/L)	16	20	24
Yeast extract (g/L)	8	10	12
Fe ₂ (SO ₄) ₃ (mmol/L)	1.2	1.6	2.0
Adenosine (g/L)	1.5	2	2.5

values of the cordycepin titer and the biomass concentration after 5 days of fermentation were compared with the predicted values.

Determination of mycelia biomass and cordycepin concentration

The fermentation liquid was centrifuged at 8000 rpm for 10 min at room temperature. The precipitated mycelia were washed twice with distilled water and freeze-dried on a Christ Alpha 2–4 LDD plus freeze dryer (Osterode, Germany) at $-55\text{ }^{\circ}\text{C}$ and 1.2×10^4 mpa for 48 h to obtain the mycelia dry mass. The determination of cordycepin concentration was carried out by HPLC with an Agilent 1260 system equipped with a Unitary C18 column (4.6 mm \times 250 mm) and a UV detector at 259 nm at $30\text{ }^{\circ}\text{C}$, gradiently eluted with ultrapure water (A) and methanol (B) at a flow rate of 0.8 mL/min in a process of 0–4 min, 15% B; 4–8 min, 20% B; 8–12 min, 25% B; 12–16 min, 20% B; and 16–20 min, 15% B [22]. The retention time of the standard cordycepin was 9.8 min. The fermentation broth was re-dissolved in 50% methanol for LC–MS analysis (Model 2020, Shimadzu, Kyoto, Japan).

Statistical analysis

All of the samples were analyzed in triplicate, and mean values were calculated with standard deviations of $<4\%$. The analysis of variance (ANOVA) was performed to evaluate

the significance of the model and coefficients. The results were analyzed by Design Expert software (Version 8.0.5, Stat-Ease Inc., Minneapolis, MN, USA), and fitted with a quadratic regression model established for variable response, as described by Eq. (4):

$$Y = b_0 + \sum b_i X_i + \sum b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (4)$$

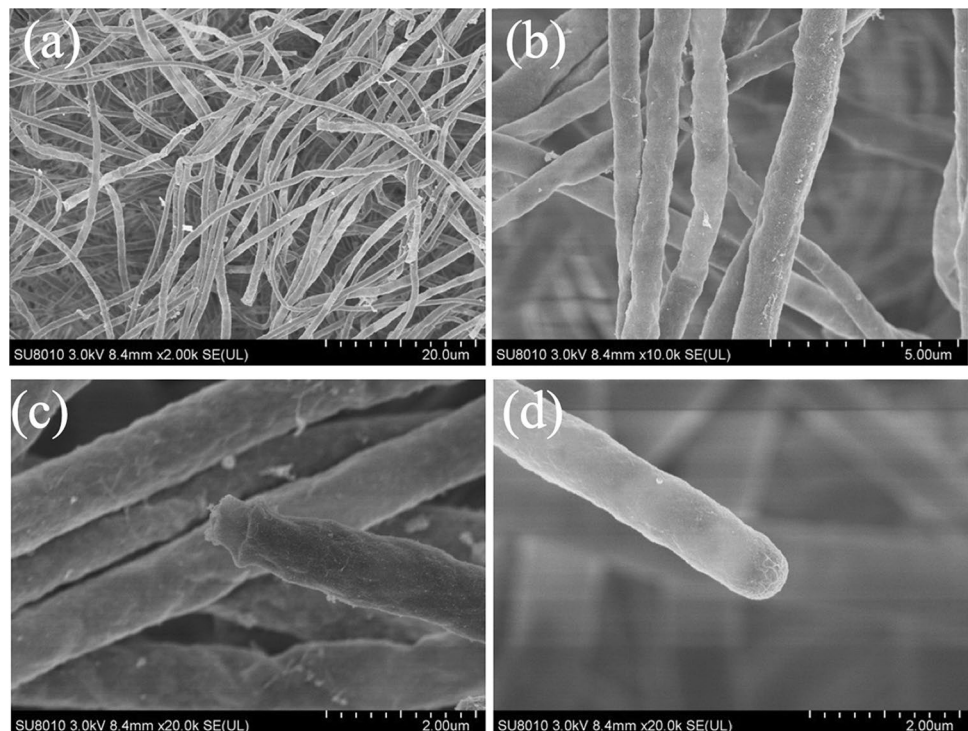
where Y is the predicted results, X_i and X_j for the independent variables of the coded value; b_0 is intercepted; I is the linear coefficient; b_{ii} is square coefficient; b_{ij} ($i \neq j$) is interaction coefficient [23].

Results and discussion

Isolation and identification of wild strain *Paecilomyces hepiali* OR-1

A single colony with a relatively high cordycepin titer was isolated and named as OR-1. The OR-1 colonies on PDA plate were white, flat, villous and dense with a neat edge in the early growth stage, and the color of the colony surface turned into orange, the hyphae were developed and raised in the middle with extended incubation time to the average size of 1.0 cm in diameter (Fig. S1). The morphology of OR-1 was also investigated by ESEM through which typical characteristics of aerial hyphae and spores were observed (Fig. 2). The hyphae of OR-1 were smooth and branched

Fig. 2 Mycelia and spores of *Paecilomyces* sp. OR-1 by ESEM **a** mycelia observation chart magnified 2,000 times; **b** is enlarge by 8000 times; **c, d** are enlarged views of the mycelium by 20,000 times



with a diameter of $\sim 2.0 \mu\text{m}$ (Fig. 2d). Biolog analysis of metabolic fingerprints showed that OR-1 could make good use of 41 carbon sources, while 45 sources could not be used, and 9 sources were in the borderline (Table S1). According to the new model bacteria category proposed by the state key laboratory of microbiology of the Chinese Academy of Sciences in 2015, strain OR-1 was preliminarily identified as *Paecilomyces* genus.

To further identify these isolated fungi, 18S rDNA sequencing and BLAST were performed revealing that the isolated strain shares a 100% identity of 560 bp to *Paecilomyces hepiali* (KX237743.1), and a 72.4% identity to *Hirsutella sinensis* (Fig. 3). Based on the above physiological and biochemical characteristics, the isolated strain was identified as *Paecilomyces hepiali* OR-1. In addition, the active component from the fermentation broth of *P. hepiali* OR-1 was confirmed by LC–MS referring to the standard cordycepin (MS(ESI⁻) $m/z = 252.02$) (Fig. 4).

Fermentation of wild strain *P. hepiali* OR-1 for cordycepin production

The submerged fermentation of *P. hepiali* OR-1 was performed for 144 h at 25 °C with a final cordycepin titer of 0.26 mg/g, hard to meet the industrial production requirement. In the first 84 h, the mycelium accumulation was in logarithmic growth from 3.3 g/L to 10.0 g/L (Fig. 5). During the subsequent stable growth period, dry mycelium weight maintained of $\sim 11.4 \text{ g/L}$. Correspondingly, the cordycepin content started to increase significantly from 36 h until 84 h, accumulated from 0.05 mg/g to 0.25 mg/g. Though the dry mycelium weight increased by 1.8 g/L to the maximum of 11.8 g/L during the extended fermentation period (84–144 h), the cordycepin content increased slightly by 0.1 mg/g to the highest value of 0.26 mg/g. Further breeding mutagenesis would be performed on this wild strain *P. hepiali* OR-1 to obtain a high-cordycepin production strain as the current cordycepin titer of *P. hepiali* OR-1 is hard to satisfy the industrial production requirement.

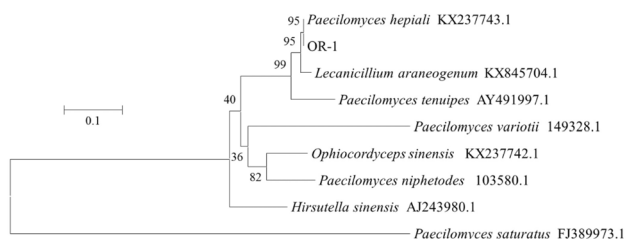


Fig. 3 The phylogenetic tree for *Paecilomyces* sp. OR-1 and related strains based on the 18S rDNA sequence. Numbers in parentheses are accession numbers of published sequences. Bootstrap values were based on 1000 replicates

⁶⁰Co γ -ray and ultraviolet irradiation on *P. hepiali* OR-1

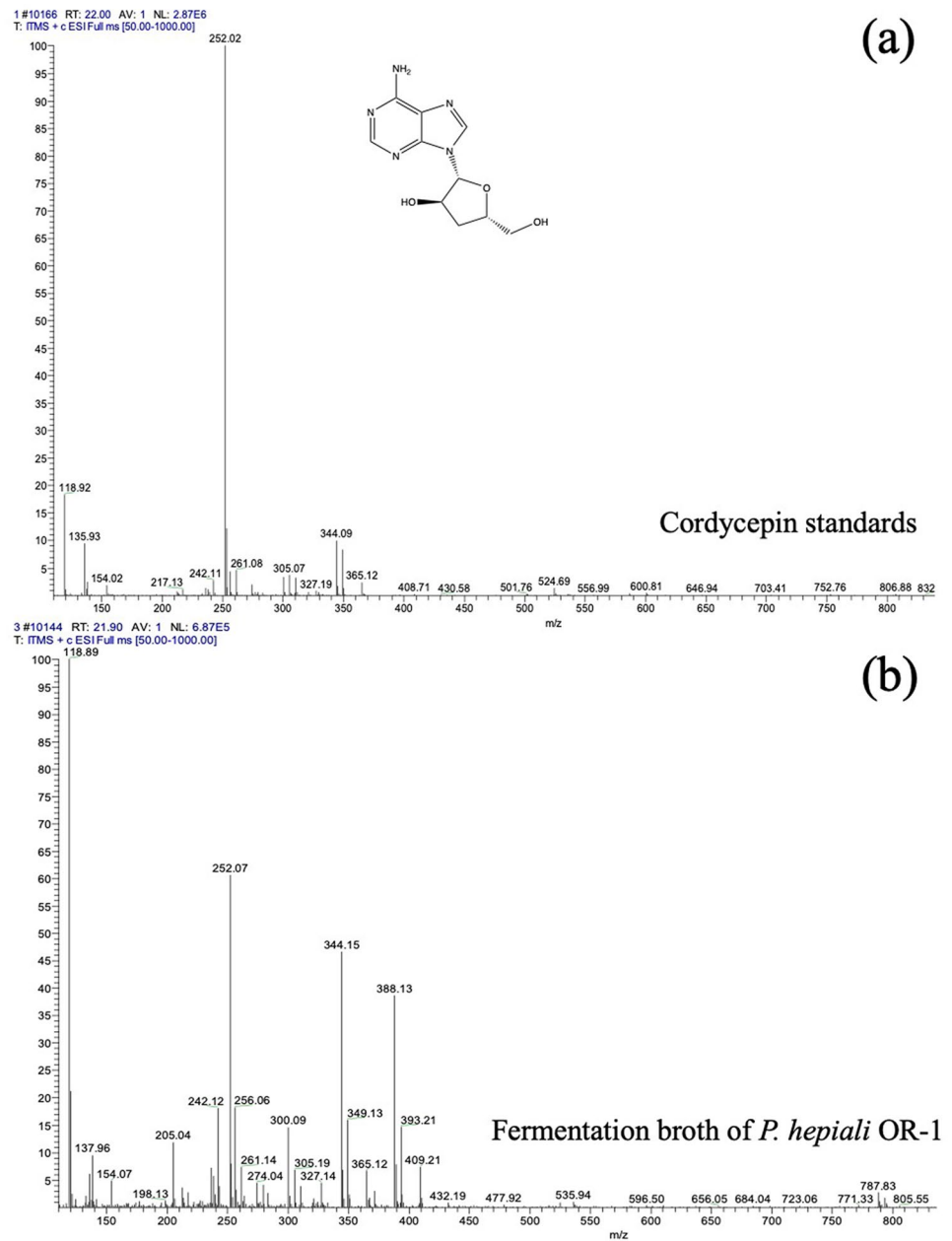
To improve the cordycepin production of the isolated strain *P. hepiali* OR-1, ⁶⁰Co γ -ray and UV irradiation were employed to generate mutant libraries. The suitable irradiation dose of ⁶⁰Co γ -ray on mycelia varies substantially among different species, for example, 1.5 kGy was selected for the mycelia of *Agaricus brasiliensis* strain with 40.3% higher biological efficiency and 14.7% higher crude protein content [11]. The appropriate dose for *P. hepiali* OR-1 was examined of 0, 100, 200, 400, 600, 800 and 1000 Gy showing a death rate of 0, 18.4, 35.0, 51.7, 78.6, 95.2 and 100% respectively. The positive rates were calculated as well of 0, 5.0, 4.3, 5.0, 6.4 and 3.1% correspondingly. Therefore, 600 Gy was selected as the median lethal dose with a lethality rate of 78.6% and a positive mutation rate of 6.4%. A mutant strain with a cordycepin yield of 0.60 mg/g_{DCW} was obtained as M1 from 17 positive strains, which is 2.3-fold to that of the wild strain. UV irradiation was subsequently applied on M1 with a 20 min irradiation time, accompanying a lethality rate of 76.4% and a positive mutation rate of 5.98%. Finally, a mutant strain exhibited an average cordycepin yield of 0.61 mg/g_{DCW} was named as ZJB18001, and stored at the China Center for Type Culture Collection (CCTCC M 2018091, Wuhan, China). The adopt of UV irradiation was not as effective as ⁶⁰Co γ -ray irradiation which increased by 0.34 mg/g_{DCW} in this study, but still worth trial as it is a simple and fast approach with satisfied results in many other strains [24, 25].

The best mutant strain ZJB18001 was compared with the wild strain *P. hepiali* OR-1 in morphology, physiological and biochemical characteristics. The single colonies of both strains showed similar morphology of white and villous, and white globular cell clusters formed in liquid medium as well. ZJB18001 was able to use 47 kinds of carbon sources, 44 kinds of which were invalid, and 4 kinds of weak usability (Table S1), indicating that the mutant strain showed wider substrate spectrum than the wild strain. The HPLC result of the fermentation broth of mutant strain ZJB18001 was shown in Fig. S3 indicating a complicated composition and a cordycepin peak at 22 min. The improvement of cordycepin titer in the fermentation media might be relevant to the chromosomal aberration or genetic mutation of key enzymes in the metabolic pathway of cordycepin synthesis in ZJB18001 after exposure to irradiation, however, genome sequencing of ZJB18001 should be further conducted to explore the underlying mechanism of cordycepin enhancement.

Single-factor optimization of medium composition

As the limited knowledge of nutrient requirements of mutant strain *P. hepiali* ZJB18001, medium optimization

Fig. 4 Mass spectrometry results of cordycepin standards (a) and fermentation broth of *Paecilomyces hepiali* OR-1 (b)



is a necessary step to improve the cordycepin content concerning the factors such as carbon source, nitrogen source, heavy metal content and precursor content. As a result, peanut oil was favorable for mycelia growth and cordycepin production among nine carbon sources by 7% enhancement in comparison with glucose (Fig. 6a). The yields of mycelia and cordycepin were most increased by yeast extract with 10.4% and 6% respectively, comparing to peptone (Fig. 6b). The optimal carbon and nitrogen sources improved the cordycepin titer from 0.61 mg/g to 0.74 mg/g indicating that *P. hepiali* ZJB18001 favors the acidic condition as peanut oil offers a relatively acidic environment. Metal ions have been reported to be participated in the regulation of

activities of enzymes, and to change the permeability of cell membrane, thus influencing the metabolites out pumping [26]. The cordycepin titer was improved by 9.5% to the value of 0.81 mg/g with the addition of 4 mmol/L metal ion Fe^{3+} (Fig. 6c). Addition of 2 g/L adenosine enabled the cordycepin increasement to 0.93 mg/g by 13.5% and dry mycelia weight by 16.3% to the value of 18.2 g/L (Fig. 6d), confirming that adenosine is a key secondary metabolite in *P. hepiali* ZJB18001, the same as the synthetic pathway of cordycepin in *C. sinensis* (Fig. 7). Lin et al. [27] demonstrated that 5'-nucleotidase participated in cordycepin synthesis, hexokinase, and glucose phosphate isomerase in *Hirsutella sinensis* by real-time

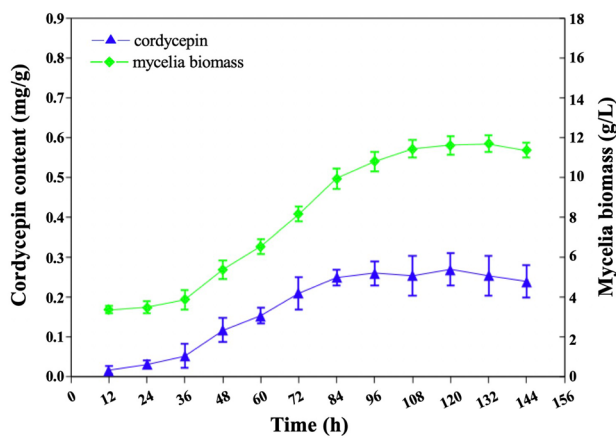


Fig. 5 Fermentation curve of wild strain *Paecilomyces hepiali* OR-1 in a 5-L fermenter

PCR, accompanying with significant 15.0, 5.3-, and 3.9-fold up-regulation, respectively. Pang et al. [28] speculated 3',5'-cyclic-nucleotide phosphodiesterase might be related

with cordycepin biosynthesis in *Paecilomyces hepiali* by transcriptome analysis. The metabolic pathway indicated that precursor substances of cordycepin might be as 3'-AMP synthesized from 2',3'-cyclic AMP, which was a by-product from mRNA degradation of *C. militaris* (Fig. 7b) [9, 29]. In sum, peanut oil, yeast extract, $Fe_2(SO_4)_3$ and adenosine were confirmed to be beneficial for cordycepin production and further optimization was carried out according to the response surface methodology.

Culture medium screening for cordycepin production enhancement

The Box-Behnken design-based experiments with four factors in three levels were performed to select the most effective culture medium specifically for *P. hepiali* ZJB18001 (Table 2). The steepest ascent experiment results showed the center points of four factors were of peanut oil 20 g/L, yeast extract 10 g/L, $Fe_2(SO_4)_3$ 1.6 g/L, and adenosine 2 g/L. The regression coefficients *F* value, *p* value and standard error were summarized and selected for ANOVAs of the

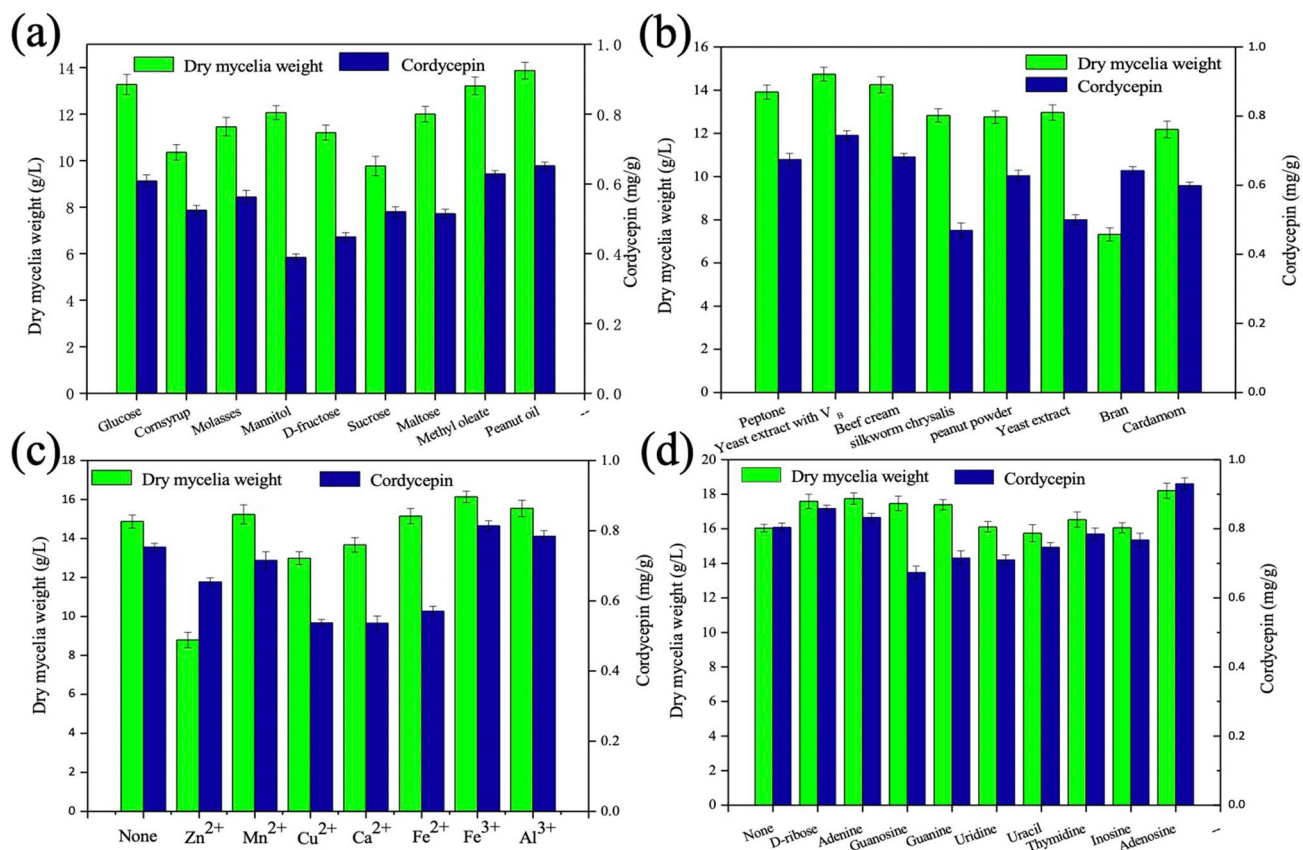


Fig. 6 Single-factor optimization of medium composition for the mutant strain *Paecilomyces hepiali* ZJB18001. **a** effects of carbon sources on mycelia mass and cordycepin production; **b** effects of nitrogen sources on mycelia mass and cordycepin production; **c**

effects of metal ion on mycelia mass and cordycepin production; **d** effects of precursor substance on mycelia mass and cordycepin production

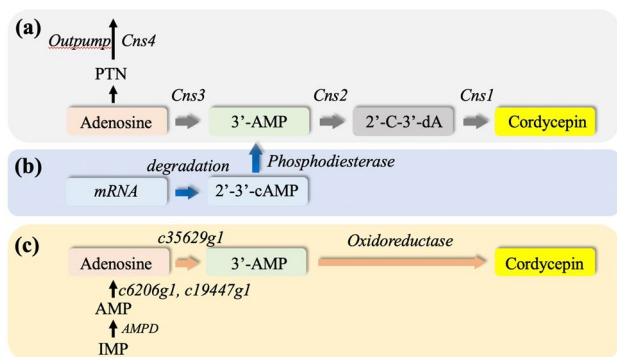


Fig. 7 The possible synthetic routes of cordycepin. **a**, **b** are the synthetic pathway found in *C. militaris* and *C. kyushensis*; **c** is the synthetic pathway found in *C. cicadae*

model to analyze the relationship between the cordycepin yield and each variable (Table S2). According to the 3D response surface curve which is a symmetrical mound shape with a coordinate axis, changes on cordycepin yield could be observed by small variations on each factor concentration (Fig. 8), indicating the variables of square interaction (X_1^2 , X_2^2 , X_3^2 , X_4^2) were all significant on the cordycepin yield. The values of F , R^2 , R^2_{Adj} , R^2_{Pred} , C.V.% and Adeq Precision were 23.78, 0.96, 0.92, 0.78, 2.35% and 17.42, respectively, for cordycepin yield (Y). The p value is less than 0.0001 implying the interaction between each factor was significant, and this model can be used to guide the optimization of the mutant strain ZJB18001.

By linear regression quadratic polynomial fitting on the experimental data, the following second-order polynomial Eq. (5) was established to explain the production of cordycepin:

Table 2 Experimental design and results of the Box-Behnken design

Runs	Coded values and real values (g/L)				Cordycepin titer (mg/g)	
	X_1 (peanut oil)	X_2 (yeast extract)	X_3 ($\text{Fe}_2(\text{SO}_4)_3$)	X_4 (adenosine)	Experimental	Predicted
1	16 (-1)	8 (-1)	1.6 (0)	2 (0)	0.71 ± 0.21	0.77
2	24 (1)	8 (-1)	1.6 (0)	2 (0)	0.85 ± 0.32	0.81
3	16 (-1)	12 (1)	1.6 (0)	2 (0)	0.82 ± 0.20	0.74
4	24 (1)	12 (1)	1.6 (0)	2 (0)	0.82 ± 0.14	0.88
5	20 (0)	10 (0)	1.2 (-1)	2.5 (1)	0.84 ± 0.17	0.89
6	20 (0)	10 (0)	2 (1)	2.5 (1)	0.74 ± 0.23	0.71
7	20 (0)	10 (0)	1.2 (-1)	1.5 (-1)	0.60 ± 0.14	0.66
8	20 (0)	10 (0)	2 (1)	1.5 (-1)	0.86 ± 0.18	0.81
9	16 (-1)	10 (0)	1.6 (0)	2.5 (1)	0.84 ± 0.16	0.79
10	24 (1)	10 (0)	1.6 (0)	2.5 (1)	0.72 ± 0.24	0.67
11	16 (-1)	10 (0)	1.6 (0)	1.5 (-1)	0.63 ± 0.19	0.68
12	24 (1)	10 (0)	1.6 (0)	1.5 (-1)	0.83 ± 0.20	0.87
13	20 (0)	8 (-1)	1.2 (-1)	2 (0)	0.63 ± 0.16	0.67
14	20 (0)	12 (1)	1.2 (-1)	2 (0)	0.82 ± 0.31	0.86
15	20 (0)	8 (-1)	2 (1)	2 (0)	0.85 ± 0.25	0.79
16	20 (0)	12 (1)	2 (1)	2 (0)	0.78 ± 0.27	0.71
17	16 (-1)	10 (0)	1.2 (-1)	2 (0)	0.66 ± 0.16	0.62
18	24 (1)	10 (0)	1.2 (-1)	2 (0)	0.70 ± 0.13	0.77
19	16 (-1)	10 (0)	2 (1)	2 (0)	0.83 ± 0.23	0.87
20	24 (1)	10 (0)	2 (1)	2 (0)	0.76 ± 0.14	0.71
21	20 (0)	8 (-1)	1.6 (0)	2.5 (1)	0.90 ± 0.34	0.84
22	20 (0)	12 (1)	1.6 (0)	2.5 (1)	0.72 ± 0.15	0.75
23	20 (0)	8 (-1)	1.6 (0)	1.5 (-1)	0.62 ± 0.17	0.67
24	20 (0)	12 (1)	1.6 (0)	1.5 (-1)	0.77 ± 0.24	0.72
25	20 (0)	10 (0)	1.6 (0)	2 (0)	0.99 ± 0.15	0.91
26	20 (0)	10 (0)	1.6 (0)	2 (0)	0.98 ± 0.16	0.91
27	20 (0)	10 (0)	1.6 (0)	2 (0)	0.99 ± 0.17	0.91
28	20 (0)	10 (0)	1.6 (0)	2 (0)	0.97 ± 0.20	0.91
29	20 (0)	10 (0)	1.6 (0)	2 (0)	0.97 ± 0.11	0.91

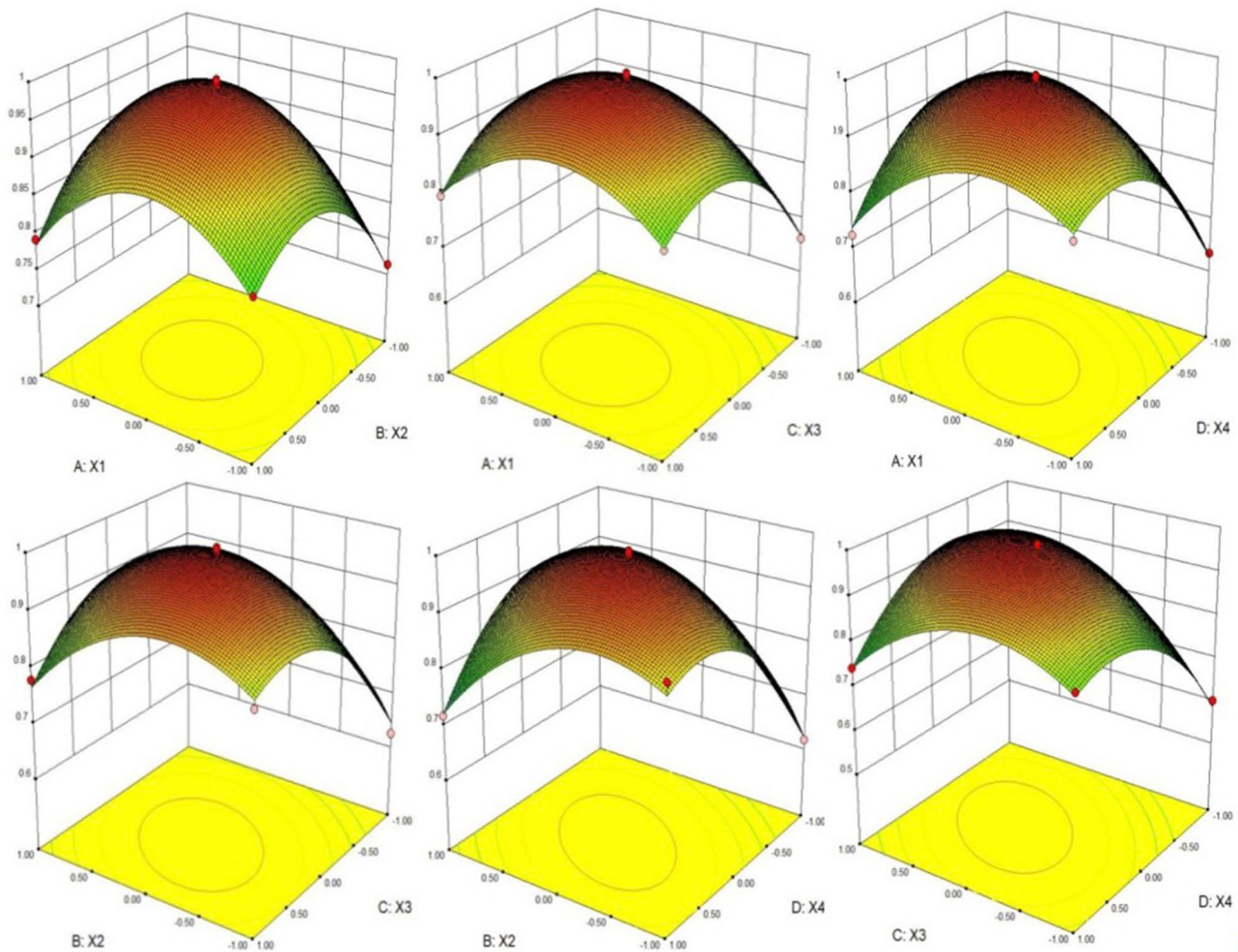


Fig. 8 3-D response surface curves of the cordycepin production of *Paecilomyces hepiali* ZJB18001 as a function of yeast extract powder and peanut oil (a), yeast extract powder and Fe³⁺ (b), yeast extract

powder and adenosine (c), peanut oil and Fe³⁺ (d), peanut oil and adenosine (e), Fe³⁺ and adenosine (f)

$$\begin{aligned}
 Y = & 0.98 + 0.022 * X_1 + 0.017 * X_2 + 0.044 * X_3 \\
 & + 0.034 * X_4 - 0.036 * X_1 * X_2 - 0.037 * X_1 \\
 & * X_3 - 0.079 * X_1 * X_4 - 0.067 * X_2 * X_3 \\
 & - 0.094 * X_2 * X_4 - 0.090 * X_3 * X_4 - 0.099 * X_1^2 \\
 & - 0.096 * X_2^2 - 0.11 * X_3^2 - 0.11 * X_4^2 \quad (5)
 \end{aligned}$$

where *Y* represents the predicted response of cordycepin content (mg/g) of mutant ZJB18001, *X*₁, *X*₂, *X*₃, and *X*₄ are the coded values for peanut oil, yeast extract, Fe₂(SO₄)₃ and adenosine, respectively.

The model was validated by conducting experiments in triplicates under the predicted medium: peanut oil 21 g/L, yeast extract 9.8 g/L, Fe₂(SO₄)₃ 4.68 mmol/L and adenosine

2.14 g/L. A mean cordycepin content of 0.96 mg/g_{DCW} was obtained, representing a good agreement with the predicted value of 0.98 mg/g_{DCW}. These results confirmed the validity and precision of the constructed model, and the accumulation amount of cordycepin was greatly increased by 1.57-fold comparing to that with the original medium.

Besides the high cordycepin production of *P. hepiali* ZJB18001 in comparison with reported strains (Table 3), such as *C. militaris* [14] and *Hirsutella sinensis* [27, 30], the cordycepin production of 0.96 mg/g by ZJB18001 in the optimized medium was threefold to that from *Hirsutella sinensis* [27]. *P. hepiali* is advantageous in a 5-day cultivation time, whereas other strains need 8–20 days, which is favored by industry in the aspect of cost reduction and time-saving.

Table 3 Summary of cordycepin production, culture medium and cultivation conditions reported in recent years

Strain	Method	Medium composition (g/L)				Cordycepin production in the broth (g/L)	Culture condition		Ref
		Carbon source	Nitrogen source	Metal ion	Precursor		Temp(°C)	Time (Day)	
<i>Cordyceps militaris</i>	Submerged culture	Glucose 86.2	Yeast extract 93.8	1/10 Vogel's medium	–	14.3	25	20	[14]
<i>Hirsutella sinensis</i>	Submerged culture	Glucose 10	Molasses 10, silkworm chrysalis powder 5, soybean cake powder 10, yeast extract 5	MgSO ₄ 0.1, KH ₂ PO ₄ 0.2	–	3.85 (0.35 mg/g)	16	12	[27]
<i>Hirsutella sinensis</i>	Submerged culture	Glucose 10	Molasses 10, silkworm chrysalis powder 5, soybean cake powder 10, yeast extract 5	MgSO ₄ 0.1, KH ₂ PO ₄ 0.2	Adenosine-5'-monophosphate 4	16.35 (1.09 mg/g)	16	12	[27]
<i>Cordyceps militaris</i> CICC 14,014	Static culture	Glucose 40 Peanut oil 20	Tryptone 10, yeast extract 6	MgSO ₄ ·7H ₂ O 0.5, K ₂ HPO ₄ ·3H ₂ O 0.5, KH ₂ PO ₄ 0.5	Adenine 1.6	5.29	25	20	[19]
<i>Cordyceps militaris</i> KYL05	Submerged culture	Glucose 20	Casein hydrolysate 20	K ₂ HPO ₄ ·3H ₂ O 0.2, KH ₂ PO ₄ 0.1, MgSO ₄ ·7H ₂ O 0.2	–	0.45	25	6	[15]
<i>Paecilomyces hepiali</i>	Submerged culture	Glucose 20	Peptone 5	KH ₂ PO ₄ 1, MgSO ₄ 0.5	–	3.11 (0.26 mg/g)	25	5	This study
<i>Paecilomyces hepiali</i> ZJB18001	Submerged culture	Peanut oil 20	Yeast extract 10	KH ₂ PO ₄ 1, MgSO ₄ 0.5, Fe ₂ (SO ₄) ₃ 4 mmol/L	Adenosine 2	8.10 (0.61 mg/g)	25	5	This study
<i>Paecilomyces hepiali</i> ZJB18001	Submerged culture	Peanut oil 21	Yeast extract 9.8	KH ₂ PO ₄ 1, MgSO ₄ 0.5, Fe ₂ (SO ₄) ₃ 4.68 mmol/L	Adenosine 2.14	17.86 (0.96 mg/g)	25	5	This study

Conclusions

The aim of this study was to screen a strain that are capable of producing high content of cordycepin as an alternative to *C. sinensis* in an industrial-scale production. Irradiation mutagenesis implemented on the wild strains was a common but effective approach for the enhancement of bioactive metabolites that have been used for

decades in industry when the genetic background of a certain strain is not clear yet. The results provide us a combined approach of ⁶⁰Co γ-ray and ultraviolet irradiation and medium screening to improve the productivity of bioactive compounds by strains. Further efforts such as genome and transcriptome analysis should be made to discover the mechanism behind the cordycepin production enhancement that could be possibly transferred to other

strains with high cordycepin yield but a slow growth rate for metabolic engineering.

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Declarations

Conflict of interest Authors have no declarations of conflict of interest.

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