VNU Journal of Science: Natural Sciences and Technology
Journal homepage: https://js.vnu.edu.vn/NST

Original Article

Effects of Culture Conditions on Growth and Cyclooligomer Depsipeptides Biosynthesis of *Cordyceps* sp. CPA14V

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Received 17 July 2021
Revised 16 August 2021; Accepted 21 August 2021

Abstract: *Cordyceps* sp. CPA14V was isolated from insect-fungi sample that was collected from Copia - Son La Nature Reserve. The strain was able to biosynthesize cyclooligomer depsipeptides (CODs). This study aimed to identify *Cordyceps* sp. CPA14V to species using DNA sequence analysis and to estimate the effects of carbon, nitrogen, pH of culture broth on its growth and COD producing capacity. The results showed that the studied strain was *Cordyceps cateniannulata* CPA14V. This is the first record of the species in Vietnam. Glucose, yeast extract and pH=8.0 were the most suitable for growth and CODs synthesis of *C. cateniannulata* CPA14V. This is also the first report of CODs produced by a *C. cateniannulata*.

Keywords: *Cordyceps cateniannulata*, cyclooligomer depsipeptides, carbon source, nitrogen source, pH.

1. Introduction

Enniatin A from *Fusarium orthocera* var. *enniatinum* was structurally the first cyclooligomer depsipeptides (CODs) determined [1]. Since then, some other fungi have been recoded of producing CODs. There have been over 14 fungal genera with COD producing capacity but commonly species of *Acremonium, Beauveria, Cordyceps, Fusarium, Hirsutella, Isaria, Paecilomyces, Verticillium*, which normally parasited insects [2-4].

CODs are biosynthesized outside the ribosome by complex enzymes (nonribosomal peptide synthetases (NRPSs) [5]. CODs have a broad spectrum of biological activities, which is considered as a potential new source of natural materials for application in medicine [3]. CODs producing capacity is highly dependent on culture conditions [6]. Especially, carbon, nitrogen, and pH of culture broth have been shown to play a very important role in the growth and COD producing capacity of fungi [7-9]. Therefore, optimizing the culture conditions is essential in the production of CODs.

In fungi, the average ITS region is about 527-700 bp long, which is a rapidly evolving

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https://doi.org/10.25073/2588-1140/vnunst.5273

region that can vary in sequence and length. Therefore, ITS is widely used in fungal taxonomy as well as for phylogenetic assessment at species level [10]. However, for many groups of fungi, additional sequences were needed for a better phylogenetic resolution, and some LSU, RPB2, EF, Actin,… were commonly used sequences. When combined ITS with other barcoding DNA such as *Rpb*1, LSU, the correct identification probability index (PCI index) to the species increased to 0.78 [11].

Vietnam has been considered as a hotspot in biodiversity. However, the understanding of fungal diversity and applications is limited. There have been some studies of *Cordyceps* diversity on insects [12, 13], one study on Triterpenoit and Steroit from *Isaria japonica* [14] but none of the published studies in Vietnam was done on CODs production. In this study, we carried out identification *Cordyceps* sp. CPA14V by *DNA* sequence analysis and effects of pH, carbon, nitrogen sources on CODs biosynthesis of the strain.

2. Experiments

2.1. Chemicals and Materials

2.1.1. Chemicals

Czapek-Dox (CzD): sucrose 30 g/L; $NaNO_3$ 3.5 g/L; K_2HPO_4 1.5 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $FeSO_4.7H_2O$ 0.1 g/L, KCl 0.5 g/L.

Sabouraud (SBR): glucose 40 g/L; pepton 10 g/L.

Carbon sources: sucrose, glucose, lactose, soluble starch, galactose, and manitose.

Nitrogen sources: $NaNO_3$, Yeast extract, Pepton, Meat extract, KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3, and Monosodium glutamate.

2.1.2. Materials

The CPA14V insect-fungus sample was collected from Copia - Son La Nature Reserve on 24/12/2016. Fungal strain was isolated from the sample.

2.2. DNA Extraction

The strain was cultured on Sabouraud broth for 48 hours and then centrifuged at 10000 rpm for 5 minutes at 4°C. 300 mg of fungal biomass was collected and put into and a sterile 2 ml eppendorf tube. SDS buffer (600 µl) was added into the tube. Fungal mycelia were broken by ultrasonic for 10-30 seconds and then incubated at 65°C for 60 minutes. Sodium acetate (3M, pH 5.2, 300 µl) then added and centrifuged at 13000 rpm for 20 minutes at 4°C. The supernatant was transferred to a fresh 1.5 ml Eppendorf tube and the same volume of isopropanol was added. The mixture was centrifuged at 13000 rpm for 15 minutes, at 4°C. The supernatant was discarded. The precipitate was then washed with 500 µl of 70% ethanol for two times and left to dry for 10-15 minutes at room temperature. TE buffer (pH 8, 50 µl) and 3 µl of RNase (10 mg/ml) were added and incubated at 60°C for 30 minutes. The DNA was then stored 0-4°C for immediate uses or at -20°C for further uses [15].

2.3. PCR reaction

The primer pairs used in this study were ITS4-ITS5 [16], LROR-LR7 [17], and Crpb1A-RPB1Cr [18]. The Dream Taq PCR Master Mix (2X) (Thermo Fisher Scientific, USA) was used for the amplification of the fragments with a thermal cycle as followed: Initial denaturation 94°C for 3 minutes, 30 cycles of 94°C for 1 minute, 52°C for 50 seconds, 72°C for 1 minute, final extension of 72°C for 10 minutes. Characterisation of PCR products was done via agarose gel electrophoresis on a 0.8% agarose gel containing ethidium bromide as the staining agent.

2.4. DNA Sequences analysis

PCR products were sequenced by First BASE Company - Singapore. Sequences were checked using BioEdit 7.2 [19] and then

Blasted using nBlast (NCBI). Related sequences were chosen for analysis. The sequences were aligned and analysed using ClustalX1.83 [20]. The phylogenetic tree was visualized by Mega X software [21].

2.5. Effect of Carbon Source on Growth and CODs Biosynthesis of Cordyceps sp. CPA14V

Czapek-Dox medium (sucrose 30 g/L; $NaNO_3$ 3.5 g/L; K_2HPO_4 1.5 g/L; $MgSO_4.7H_2O$ 0.5 g/L; $FeSO_4.7H_2O$ 0.1 g/L, KCl 0.5 g/L) was used as base medium where the same amount of different sugars (sucrose, glucose, lactose, soluble starch, galactose, and mannitol) were added. The strain (0.5 ml) was poured into 100 ml conical flask containing 25 ml of medium and then incubated at 25°C, rotating at 150 rpm for six days. Cell dry weight (CDW) were abtained to evaluate fungal growth and determine its CODs amounts.

2.6. Effect of Nitrogen Source on the Growth and CODs Biosynthesis of Cordyceps sp. in CPA14V

Czapek-Dox with the most suitable carbon source was used as base medium and the same amount (3.5 g/L) of different nitrogen containing compounds ($NaNO_3$, Yeast extract, Pepton, Meat extract, KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, NH_4HCO_3, and Monosodium glutamate) was added. The strain (0.5 ml) was poured into 100 ml conical flask containing 25 ml of medium and then incubated at 25°C, rotating at 150 rpm for six days. CDW were abtained to evaluate fungal growth and determine its CODs amounts.

2.7. Effect of Medium's pH on the Growth and CODs Biosynthesis of Cordyeps sp. CPA14V

Czapek-Dox with the most suitable carbon and nitrogen sources was used and adjusted pH to different value from 3.0 to 8.0 with stepwise of 0.5. The strain (0.5 ml) was poured into 100 ml conical flask containing 25 ml of medium and then incubated at 25°C, rotating at 150 rpm for six days. CDW were abtained to evaluate fungal growth and determine its CODs amounts.

2.8. CDW Determination

The 2 ml Eppendorf tubes were numbered, dried to constant mass, and weighed to determine the initial mass (M_o) of each tube. Fungal samples (1.5 ml) were taken into the tubes and then centrifuged at 12000 rpm, 4°C for 5 minutes. The supernatant was removed. Another 1.5 ml of culture broth was added and centrifuged. The precipitated biomass was washed with distilled water, then lyophilized to constant weight. The freeze-dried weight of the tube with biomass was M_1. Each sample was repeated three times. The CDW were calculated by the formula: $CDW\ (g/L) = (M_1 - M_o)/3*1000$.

2.9. CODs Quantification

The concentration of CODs accumulated in cells was determined by high-performance liquid chromatography (HPLC) using a BDS-C18 hypersil column and acetonitrile-water solvent system. Analysis was performed at a flow rate of 0.3 ml/minute with a water-acetonitrile gradient, starting from acetonitrile-water (15:85) to 100% acetonitrile for 40 minutes, maintaining 100% acetonitrile for 5 minutes, before return to starting condition for 8 minutes and equilibration for 5 minutes. Probe wavelength: 203 nm [22]. Purified Beauvericin (Sigma - USA) was used to construct the standard graph.

2.10. Data Processing

The collected data and statistics were processed by Microsoft Office Excel 2010 and SPSS 20.

3. Results and Discussion

3.1. Identification of Fungal Strains CPA14V

The sequence of *Cordyceps* sp. CPA14V was used for checking the related sequences available in the NCBI GenBank revealed that the most similar sequences were *Cordyceps* species. There were 19 sequenes of *Cordyceps*, *Isaria*, *Hypocrea*, and *Ophiocordyceps* were chosen for the analysis. *Ophiocordyceps*

sequences were used as root sequences. The analysis was one with distance methods and 1000 times of bootstraps.

The results showed that *Cordyceps* sp. CPA14V was lustered with *C. cateniannulata* with 100% of bootstraps (Figure 1) for all three genes (data of LSU and RBP1 not shown). So, the isolated fungus of this study was *C. cateniannulata CPA14V* (NCBI accession number MK634637.2).

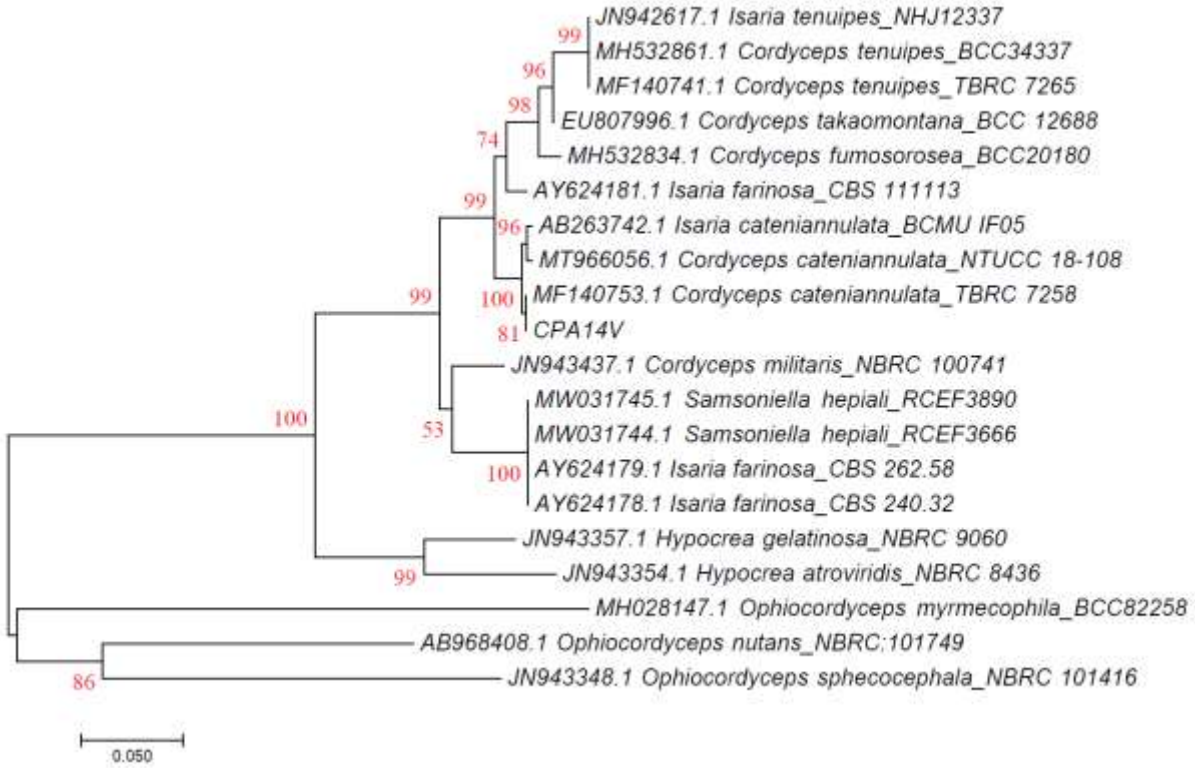

Figure 1. Phylogenetic tree of *Cordyceps* sp. CPA14V based on ITS complete sequence.

3.2. Effect of Carbon Source on Growth and CODs Biosynthesis of C. Cateniannulata CPA14V

C. cateniannulata CPA14V grew well on CzD medium with different carbon source added (Table 1). Among the tested carbon sources, glucose appeared as the most suitable carbon source for the growth and biosynthesis of CODs with a CDW of 10.2 g/L, CODs content reached 5.797 mg/g, followed by fructose and sucrose. CODs was accumulated at highest concentration when cultured on CzD with lactose as carbon source. However, the total productivity of CODs production was low as the strain grew slow on this medium. This result was similar to the published studies, showing that the ability to generate total CODs of the fungus was optimal in the medium containing glucose [7, 8].

3.3. Effect of Nitrogen Source on Growth and CODs Biosynthesis of C. Cateniannulata CPA14V

Similar to the capacity to growth in medium with different source of carbon, *C. cateniannulata* CPA14 grew and produced CODs well on medium with different sources of nitrogen (Table 2). Meat extract, Yeast extract, peptone, $NaNO_3$ and KNO_3 showed to be suitable for both growth and COD production and it was for from the rest of

nitrogen sources. The productivity of COD production of these 5 nitrogen sources were 60.330 ± 1.280 g/L, 65.789 ± 2.186 g/L, 60.771 ± 1.844 g/L, 55.961 ± 2.598 g/L, and 53.902 ± 0.619 g/L respectively. In brief, the best nitrogen for COD production should be yeast extract. The COD productivity of *C. cateniannulata* CPA14V was high compare to some other species of *Isaria* [23] and *Fusarium* [24].

Table 1. Effect of carbon source on growth and CODs biosynthesis of *C. cateniannulata* CPA14V

Carbon source (30g/L)	CDW (g/L)	CODs (mg/g)	CODs (mg/L)
Glucose	$10.205^{e} \pm 0.082$	$5.797^{d} \pm 0.078$	$59.155^{e} \pm 0.328$
Fructose	$9.395^{d} \pm 0.273$	$6.003^{de} \pm 0.039$	$56.400^{e} \pm 0.228$
Sucrose	$8.359^{c} \pm 0.232$	$5.630^{d} \pm 0.057$	$47.049^{d} \pm 0.829$
Lactose	$6.697^{b} \pm 0.109$	$6.375^{e} \pm 0.139$	$42.706^{c} \pm 1.623$
Soluble starch	$6.574^{b} \pm 0.191$	$5.631^{d} \pm 0.299$	$36.960^{b} \pm 2.997$
Glactose	$6.575^{b} \pm 0.069$	$5.118^{c} \pm 0.086$	$33.666^{b} \pm 0.922$
Mannitol	$2.463^{a} \pm 0.302$	$4.327^{b} \pm 0.021$	$10.665^{a} \pm 1.357$
Control (Non carbon source)	$2.649^{a} \pm 0.273$	$3.263^{a} \pm 0.215$	$8.587^{a} \pm 0.380$

(Comparing between formulas, in the same column, different letters (a, b, c, d, e) represent statistically significant differences with $P<0.05$)

Table 2. Effect of nitrogen source on growth and CODs biosynthesis of *C. cateniannulata* CPA14V strain

Nitrogen source (3.5 g/L)	CDW (g/l)	CODs (mg/g)	CODs (mg/L)
Meat extract	$14.403^{k} \pm 0.055$	$4.189^{c} \pm 0.104$	$60.330^{fg} \pm 1.280$
Yeast extract	$13.524^{h} \pm 0.313$	$4.863^{de} \pm 0.049$	$65.789^{g} \pm 2.186$
$NaNO_3$	$12.233^{g} \pm 0.249$	$4.967^{de} \pm 0.050$	$60.771^{fg} \pm 1.844$
KNO_3	$11.404^{f} \pm 0.151$	$4.905^{de} \pm 0.163$	$55.961^{ef} \pm 2.598$
Pepton	$10.456^{e} \pm 0.204$	$5.156^{e} \pm 0.048$	$53.902^{de} \pm 0.619$
$(NH_4)_2SO_4$	$9.457^{d} \pm 0.252$	$3.880^{b} \pm 0.099$	$36.717^{b} \pm 1.923$
NH_4NO_3	$8.739^{c} \pm 0.136$	$4.323^{c} \pm 0.088$	$37.793^{b} \pm 1.336$
MSG	$8.683^{c} \pm 0.183$	$5.700^{f} \pm 0.159$	$49.524^{cd} \pm 2.405$
NH_4Cl	$7.592^{b} \pm 0.069$	$2.817^{a} \pm 0.132$	$21.393^{a} \pm 1.189$
NH_4HCO_3	$7.413^{b} \pm 0.237$	$6.330^{g} \pm 0.126$	$46.956^{c} \pm 2.446$
Control (Non nitrogen source)	$6.853^{a} \pm 0.041$	$4.717^{d} \pm 0.057$	$32.323^{b} \pm 0.199$

(Comparing between formulas in the same column, different letters (a, b, c, d, e) represent statistically significant differences with $P<0.05$)

3.4. Effect of pH on the Growth and CODs Biosynthesis of C. Cateniannulata CPA14V

Medium pH affects the dissociation of ions, structure, and activity of enzymes, so it has a decisive influence on the growth, development, and biosynthesis of CODs. The studied strain did not grow well at medium pH value lower than 6 (Table 3). But it grew well when pH value range from 6.5 to 8 with no significant differences. However, CODs production was highest at pH 8 and

significantly different to the other. The total production of CODs at pH 8 was 50.774 ± 1.046 g/L, followed by 46.587 ± 0.429 g/L at pH 7.5. The CODs productivity was gradually reduced along with medium pH value reduction.

This result was similar to the published studies, showing that the ability to generate total CODs of the fungus was optimal in the condition of medium pH fluctuating around pH=7.0 [6].

Table 3. Effect of pH on growth and CODs biosynthesis of *C. Cateniannulata* CPA14V strain

pH	CDW (g/l)	CODs (mg/g)	CODs (mg/L)
3	$0.067^{a} \pm 0.003$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
3.5	$0.430^{ab} \pm 0.08$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
4	$0.604^{b} \pm 0.01$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
4.5	$0.358^{ab} \pm 0.041$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
5	$0.412^{ab} \pm 0.003$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
5.5	$0.595^{b} \pm 0.016$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
6	$1.258^{c} \pm 0.066$	$0^{a} \pm 0.000$	$0^{a} \pm 0.000$
6.5	$8.360^{d} \pm 0.107$	$4.963^{b} \pm 0.049$	$41.505^{b} \pm 0.116$
7	$8.270^{d} \pm 0.024$	$5.117^{b} \pm 0.130$	$42.318^{b} \pm 1.198$
7.5	$8.060^{d} \pm 0.144$	$5.783^{c} \pm 0.053$	$46.587^{c} \pm 0.429$
8	$8.200^{d} \pm 0.287$	$6.190^{d} \pm 0.090$	$50.774^{d} \pm 1.046$

(Comparing between formulas in the same column, different letters (a,b,c,d,e) represent statistically significant differences with $P<0.05$)

4. Conclusion

Cordyceps sp. CPA14V parasite on insect was identified to *Cordyceps Cateniannulata* CPA14V based on molecular anylysis. The strain grew and biosynthesized CODs well in the Czapeck-Dox medium where the carbon source was glucose (30 g/L), the nitrogen source was Yeast extract (3.5 g/L) and the pH=8.0, with the COD yield of 65.789 ± 2.186 g/L. *C. cateniannulata* was new records of fungus to Vietnam. The finding in this study was the first record of *C. cateniannulata* with ability of producing CODs.

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