Original Article

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

Nguyen Thi Thuy Van¹,², Nguyen Dinh Viet¹, Duong Minh Lam¹,*

¹Hanoi National University of Education, 136 Xuan Thuy, Cau Giay, Hanoi, Vietnam
²The People’s Police Academy, Co Nhue 2, Bac Tu Liem, Hanoi, Vietnam

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**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 orthoceras* 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 (NRPs)) [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
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 Rpb1, 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; NaNO₃ 3.5 g/L; K₂HPO₄ 1.5 g/L; MgSO₄.7H₂O 0.5 g/L; FeSO₄.7H₂O 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: NaNO₃, Yeast extract, Pepton, Meat extract, KNO₃, NH₄Cl, NH₄NO₃, (NH₄)₂SO₄, NH₄HCO₃, 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 30 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

Czapec-Dox medium (sucrose 30 g/L; NaNO₃ 3.5 g/L; K₂HPO₄ 1.5 g/L; MgSO₄.7H₂O 0.5 g/L; FeSO₄.7H₂O 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 medium and then incubated at 25°C, rotating at 150 rpm for six days. Cell dry weight (CDW) were obtained to evaluate fungal growth and determine its CODs amounts.

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

Czapec-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 (NaNO₃, Yeast extract, Pepton, Meat extract, KNO₃, NH₄Cl, NH₄NO₃, (NH₄)₂SO₄, NH₄HCO₃, and Monosodium glutamate) was added. The strain (0.5 ml) was poured into 100 ml conical flask containing 25 ml medium and then incubated at 25°C, rotating at 150 rpm for six days. CDW were obtained to evaluate fungal growth and determine its CODs amounts.

2.7. Effect of Medium’s pH on the Growth and CODs Biosynthesis of Cordyceps sp. CPA14V

Czapec-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 medium and then incubated at 25°C, rotating at 150 rpm for six days. CDW were obtained 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 (M₀) 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 M₁. Each sample was repeated three times. The CDW were calculated by the formula: CDW (g/L) = (M₁ - M₀)/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 clustered 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, NaNO₃ and KNO₃ 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 compared 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 ± 0.082    | 5.797 ± 0.078 | 59.155 ± 0.328 |
| Fructose             | 9.395 ± 0.273     | 6.003 ± 0.039 | 56.400 ± 0.228 |
| Sucrose              | 8.359 ± 0.232     | 5.630 ± 0.057 | 47.049 ± 0.829 |
| Lactose              | 6.697 ± 0.109     | 6.375 ± 0.139 | 42.706 ± 1.623 |
| Soluble starch       | 6.574 ± 0.191     | 5.631 ± 0.299 | 36.960 ± 2.997 |
| Glucose              | 6.575 ± 0.069     | 5.118 ± 0.086 | 33.666 ± 0.922 |
| Mannitol             | 2.463 ± 0.302     | 4.327 ± 0.021 | 10.665 ± 1.357 |
| Control (Non carbon source) | 2.649 ± 0.273 | 3.263 ± 0.215 | 8.587 ± 0.380 |

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 ± 0.055 | 4.189 ± 0.104 | 60.330 ± 1.280 |
| Yeast extract             | 13.524 ± 0.313 | 4.863 ± 0.049 | 65.789 ± 2.186 |
| NaNO₃                     | 12.233 ± 0.249 | 4.967 ± 0.050 | 60.771 ± 1.844 |
| KNO₃                      | 11.404 ± 0.151 | 4.905 ± 0.163 | 55.961 ± 2.598 |
| Pepton                    | 10.456 ± 0.204 | 5.156 ± 0.048 | 53.902 ± 0.619 |
| (NH₄)₂SO₄                 | 9.457 ± 0.252 | 3.880 ± 0.099 | 36.717 ± 1.923 |
| NH₄NO₃                   | 8.739 ± 0.136 | 4.323 ± 0.088 | 37.793 ± 1.336 |
| MSG                       | 8.683 ± 0.183 | 5.700 ± 0.159 | 49.524 ± 2.405 |
| NH₄Cl                     | 7.592 ± 0.069 | 2.817 ± 0.132 | 21.393 ± 1.189 |
| NH₄HCO₃                   | 7.413 ± 0.237 | 6.330 ± 0.126 | 46.956 ± 2.446 |
| Control (Non nitrogen source) | 6.853 ± 0.041 | 4.717 ± 0.057 | 32.323 ± 0.199 |

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
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±0.003 | 0±0.000 | 0±0.000 |
| 3.5 | 0.430±0.08  | 0±0.000 | 0±0.000 |
| 4   | 0.604±0.01  | 0±0.000 | 0±0.000 |
| 4.5 | 0.358±0.041 | 0±0.000 | 0±0.000 |
| 5   | 0.412±0.003 | 0±0.000 | 0±0.000 |
| 5.5 | 0.595±0.016 | 0±0.000 | 0±0.000 |
| 6   | 1.258±0.066 | 0±0.000 | 0±0.000 |
| 6.5 | 8.360±0.107 | 4.963±0.049 | 41.505±0.116 |
| 7   | 8.270±0.024 | 5.117±0.130 | 42.318±1.198 |
| 7.5 | 8.060±0.144 | 5.783±0.053 | 46.587±0.429 |
| 8   | 8.200±0.287 | 6.190±0.090 | 50.774±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 analysis. 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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