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Original Article

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

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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 synthesize 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 COD 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 depsipeptide (COD) 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]. COD producing capacity is highly depended 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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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 its/their 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 COD production. In this study, we identified *Cordyceps* sp. CPA14V by DNA sequence analysis and evaluated effects of pH, carbon, nitrogen sources on COD 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 December 24th, 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 ^oC. 300 mg of fungal biomass was collected and put into 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 ^oC for 60 minutes. Sodium acetate (3M, pH 5.2, 300 µl) then added and centrifuged at 13000 rpm for 20 minutes at 4 ^oC. 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 ^oC. 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 ^oC for 30 minutes. The DNA was then stored 0-4 ^oC for immediate uses or at -20 ^oC 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 ^oC for 3 minutes, 30 cycles of 94 ^oC for 1 minute, 52^oC for 50 seconds, 72 ^oC for 1 minute, and final extension of 72 ^oC 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 Sequence 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 COD 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 and the same amount of different sugars (sucrose, glucose, lactose, soluble starch, galactose, and mannitol) was added. The strain culture (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) was obtained to evaluate fungal growth and determine its COD amounts.

2.6. Effect of Nitrogen Source on the Growth and COD 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 cultrure (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 was obtained to evaluate fungal growth and determine its COD amounts.

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

Czapek-Dox with the most suitable carbon and nitrogen sources was used and pH was adjusted to different value from 3.0 to 8.0 with stepwise of 0.5. The strain culture (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 was obtained to evaluate fungal growth and determine its COD 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 sample (1.5 ml) was taken into the tube 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 was calculated by the formula: CDW (g/L) = $(M_1 - M_o)/3*1000$.

2.9. COD 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 was 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 Strain CPA14V

The sequence of *Cordyceps* sp. CPA14V was used for checking the related sequences available in the NCBI GenBank and it revealed that the most similar sequences were

Cordyceps species. 19 sequenes of *Cordyceps*, *Isaria*, *Hypocrea*, and *Ophiocordyceps* were chosen for the analysis. *Ophiocordyceps* sequences were used as root sequences. The analysis was done using distance method 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 RBP1was not shown). So, the isolated fungus of this study was *C. cateniannulata CPA14V* (NCBI accession number MK634637.2).

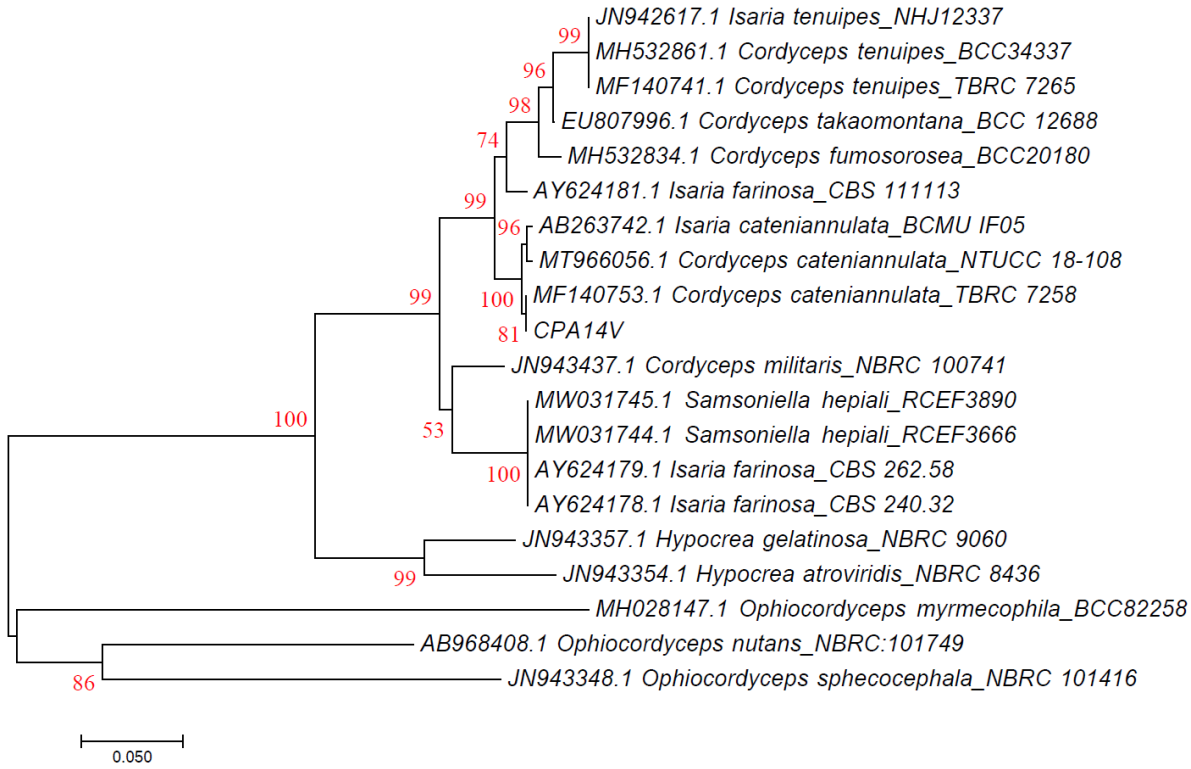

Figure 1. Phylogenetic tree of *Cordyceps* sp. CPA14V based on *ITS* complete sequence.

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

C. cateniannulata CPA14V grew well on CzD medium with different added carbon sources (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 and COD content reached 5.797 mg/g, followed by fructose and sucrose. CODs were accumulated at the highest concentration when cultivation on CzD with lactose as carbon source. However, the total productivity of CODs was low as the strain grew slow on this medium. This result was similar to the previously 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 COD 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 were suitable for both growth of *C. cateniannulata* CPA14 and COD production. The productivity of CODs using 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 source for COD production should be yeast extract. The COD productivity of *C. cateniannulata* CPA14V was higher compared to some other species of *Isaria* [23] and *Fusarium* [24].

Table 1. Effect of carbon source on growth and COD 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 among 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 COD 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 among 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 COD Biosynthesis of C. Cateniannulata CPA14V

Medium pH affects the dissociation of ions, structure, and activity of enzymes, thus it decisively influences on the growth, development, and biosynthesis of CODs. The studied strain did not grow well at medium pH value lower than 6 (Table 3). It grew well when pH value ranges from 6.5 to 8 with no significant differences. However, COD production was highest at pH 8 and

significantly different to the others. 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 COD productivity was gradually reduced along with decrease in medium pH value.

This result was similar to the previously published studies, indicating that the ability to generate total CODs of fungus was optimal at medium pH of around 7.0 [6].

Table 3. Effect of pH on growth and COD 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 among 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 synthesized CODs well in the Czapeck-Dox medium with glucose (30 g/L) as carbon source, Yeast extract (3.5 g/L) as nitrogen source and pH of 8.0, producing the CODs of 65.789 ± 2.186 g/L. *C. cateniannulata* is a new record of fungus in Vietnam. This is the first finding of *C. cateniannulata* with ability of producing CODs.

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