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Study of chemical compositions of *Cordyceps pseudomilitaris* pigments by Gas Chromatography – Mass Spectrometry (GC-MS)

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Study of mycelium growth of *C. pseudomilitaris* on PDA and MCM medium twenty one days after inoculation, colony diameter were 2.72 and 2.76 cm on PDA and MCM medium, respectively. Mycelium dry weight of *C. pseudomilitaris* in PDB and MCM medium in shaking condition showed more growth than static condition. In static condition, mycelium dry weight was 0.108 g in both medium. While, in shaking condition culturing C. *pseudomilitaris* in MCM broth showed more growth than PDB the mycelium dry weight were 0.234 and 0.166 g in MCM broth and PDB, respectively. The pigments analysis by GC-MS showed 22 compounds in shaking condition and 17 compounds in static condition. In two conditions showed eight similar compounds including Mevalonic lactone, Phenol, 1,1,3-Trimethyl-3-phenylindan, 1,2,4-Triazolidine-3,5-dione, Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), 1,4-diaza-2,5-dioxo-3-isobutyl bicycle (4.3.0) nonane, N-Methyl-2-Propyl-5-Butylpiperidine, and Phthalic acid.

Keywords: *Cordyceps pseudomilitaris*, pigments, GC-MS

Introduction

Cordyceps is one of a growing number of fungal traditional Chinese medicinal being considered as cures for modern human diseases (Russell and Paterson, 2008). The fungus represents a genus of perithecial ascomycetes (Phylum Ascomycota) classified in the Clavicipitaceae, a monophyletic group including in the order Hypocreales. The genus contains over 400 species and the anamorphs of most are unknown. *Cordyceps* are parasites of insects, often exhibiting a high degree of host specificity. Larval infection via meiotic and/or

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mitotic spores/ conidia and multiplication within the insect is from yeast like budding. However, the fungus grows through the insect by hyphae. The accumulation of the biomass eventually kills the host. The fungus ruptures the host body following over wintering and forms the sexual perithecial stroma that are connected to the dead larva below ground which grow upward to emerge above the soil surface. Various bioactive constituents from *Cordyceps* species have been reported. These include cordycepin and other antibacterial and antitumor adenosine derivatives, ophicordin, antifungal agent, and L-tryptophan (Wu *et al*., 2005). The bioactive compounds involved in the activities claimed include polysaccharides, modified nucleosides, and cyclosporine-like metabolites which are produced by this fungus and related species.

Cordyceps pseudomilitaris is an insect pathogenic fungi isolated in Thailand that infecting the immature stage of the order Lepidoptera. (Poomputsa *et al*., 1999). The morphology of this fungus is closely related to that of *C. militaris* which is known to produce several secondary metabolites including a nucleoside antibiotic, cordycepin (Isaka *et al*., 2000). This species produced two interesting metabolites groups namely ten bioxanthracenes (ES2425) and two alkenoic acids (CP-A,B). The culture broth of *C. pseudomilitaris* 4671 showed the biological activity against HIV-1 Reverse Transcriptase (RT) at IC50 73.5 ug/ml. (Plaingam, 1998). *C. pseudomilitaris* produced red pigment in culture media. Mushrooms do not contain the pigments that dominate in higher plant colours. Chlorophylls and anthocyanins are not present in fungi at all; betalains, carotenoids and other terpenoids are widespread only in some species of higher fungi (Velisek and Cejpek, 2011).

Many of the pigments of higher fungi are quinones or similar conjugated structures that are mostly classified according to the perceived biosynthetic pathways, reflecting their structure, to pigments derived from the shikimate (chorismate) pathway, the acetatemalonate (polyketide) pathway, the mevalonate (terpenoid) pathway, and pigments containing nitrogen (Gill, 2003; Hanson, 2008; Raisanen, 2009; Zhou and Liu, 2010). Various pigments and other fungi constituents show important biological activities (antioxidative, free radical scavenging, anticarcinogenic, immunomodulatory, antiviral, and antibacterial) that have generated intensive research interest (Calvia *et al*., 2003; Liu, 2006; Schuffler and Anke, 2009).

In the past, a large number of analytical tools, especially chromatography, have been used to analyze the constituents of traditional Chinese medicine (*Cordyceps* spp.) in order to control their quality and discover bioactive compounds. Today, gas chromatography–mass spectrometry (GC–MS) and other chromatographic methods have been developed for traditional Chinese medicine analysis (Deng *et al.*, 2007). GC-MS are an effective combination for

the analysis of volatile chemicals. Gas chromatography uses a carrier gas to move analytes through a coated, fused silica capillary. Separation occurs based on differential partition between the gas phase and the coating inside the capillary. GC-MS required the analyte to be vaporized in order for migration through the capillary to occur. Analytes, therefore, must be volatile or amenable to chemical derivatization to render them volatile. GC-MS has proved to be a fast and reliable approach, allowing identification of a large number of compounds (Torras-Clavevia *et al.*, 2010). In some cases, GC-MS screening of plant samples has revealed compounds with unknown MS spectra, which has resulted in the isolation and spectroscopic identification of new natural bioactive molecules (Berkov *et al.*, 2008). The compounds in the alkaloid fraction were identified by comparing their GC-MS spectra and RI with those of authentic compounds previously isolated and identified by comparing their mass spectral fragmentation with standard reference.

Hence, the present study aimed to analysis and comparison of chemical composition in red pigment of *C. pseudomilitaris* BCC 31665 in two condition including shaking and static condition.

Materials and methods

Cultivation of Cordyceps pseudomilitaris

Cordyceps pseudomilitaris BCC31665 was obtained from BIOTEC Culture Collection, National Center for Genetic Engineering and Biotechnology, Thailand. This fungi was grown on potato dextrose agar (PDA) and mushroom complete medium (MCM) in 9 cm diameter Petri dishes and incubated at 28°C, colony diameter were measure at 7, 14 and 21 days after inoculation.

Liquid culture: Pure cultures of *C. pseudomilitaris* BCC31665 was maintained on PDA plates and incubated at 28°C for 14 days and one plug (8 mm in diameter) of fungal mycelium was aseptically transferred to 100 ml aliquots of potato dextrose broth and into 100 ml aliquots of mushroom complete medium broth in 250 ml Erlenmeyer flasks. Cultures were grown at 28°C with shaking 150 rpm or incubated at static condition for 21 days. To determine the mycelia dry weight, the mycelium were filtered through filter paper (Whatman N0.1) then the mycelium were washed with distill water for 2 time and dried for 8 h at 80°C.

Analysis of chemical composition in pigments

Extraction of Fungal Pigments

C. pseudmilitaris BCC31665 was culture is the same as described above in MCM broth. To prepare the red pigment, culture filtrate of *C. pseudmilitaris* BCC31665 was extracted with ethyl acetate (1:1, v/v) for 30 min on a rotary shaker (120 rpm) at room temperature (Velmurugan *et al.*, 2010). Culture filtrate were collected only in ethyl acetate layer, then evaporated in a rotary evaporation at 60°C until dry. Acetone were added for dissolved the pigment and dried at 60°C for 1 h (Guan *et al.*, 2010). Dried pigments of *C. pseudmilitaris* BCC31665 was suspended in acetone and then filtered. The concentrations of pigment solution was adjusted to 1,000 ppm.

GC-MS Analysis

GC-MS was performed on A Rtx-5MS capillary column (30 m x 0.25 mm, I.d.) coated with 0.25μm film 5% phenyl methyl siloxane was used for separation. The column temperature was set at 45°C, then programmed at 45°C/min to 200 °C. The injection temperature was 280°C. Split injection (1 μl) with a split ratio of 1:60 was applied. The data from GC-MS were compared with NIST147.LiB and WILEY7.LIB library.

Results and discussions

Cultivation of Cordyceps pseudomilitaris

Mycelium growth of *C. pseudomilitaris* on PDA and MCM medium showed the slow growth of in both media. Twenty one days after inoculation, colony diameter were 2.72 and 2.76 cm on PDA and MCM medium, respectively (Table 1). However, the amount of pigment developed on PDA was higher than that of MCM medium. Mycelium dry weight of *C. pseudomilitaris* in PDB and MCM broth in shaking condition showed more growth than static condition. In static condition, mycelium dry weight was 0.108 g in both medium. While, in shaking condition culturing C. *pseudomilitaris* in MCM broth showed more growth than PDB the mycelium dry weight were 0.234 and 0.166 g in MCM broth and PDB, respectively (Table 2). Consistently, previous studies about growth optimization and characteristics of *C. pseudomilitaris* 4671 by comparing the cultivation medium they found YES (yeast extract sucrose) produced the maximum dry weight. At 25°C and 30°C there was a higher yield of dry biomass when liquid culture

flasks were agitated compared to static growth condition. The optimal growth temperature was 25-30°C, no growth was observed at 37°C. On solid media including Potato dextrose, Sabouraud dextrose, Malt extract, Minimum medium, and cZapek-Dox agar, at 25°C, *C. pseudomilitaris* 4671 showed slightly faster growth but produced less pigment that at 30°C. In liquid culture the pH range for growth varied depending on whether shake or static conditions were used. Under shake condition, *C. pseudomilitaris* 4671 grew in a broader initial pH rang (3-8) than under static condition (4-7) (Plaingam, 1998).

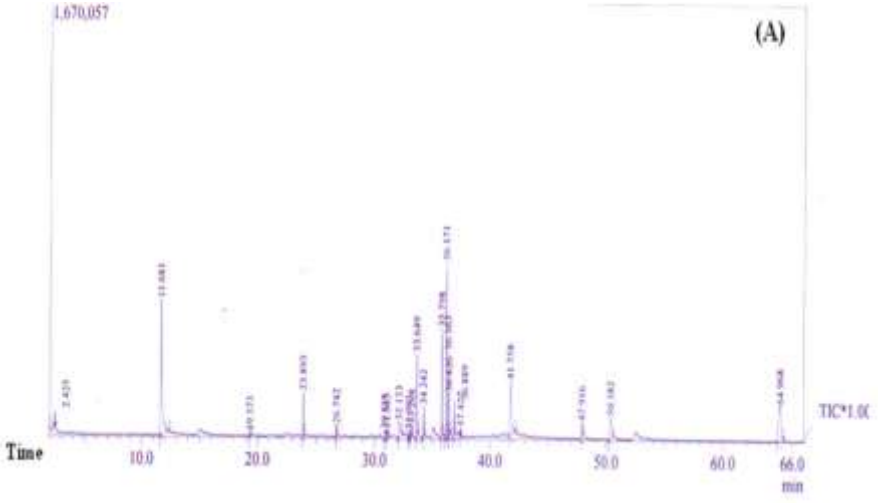
Analysis of chemical composition in pigments of C. pseudomilitaris by GC-MS

In this study we found several chemical compounds in pigment of *C. pseudomilitaris*. The pigments analysis by GC-MS showed that 22 compounds in shaking condition, while in static condition showed 17 compounds. In two conditions showed eight similar compounds including Mevalonic lactone, Phenol, 1,1,3-Trimethyl-3-phenylindan, 1,2,4-Triazolidine-3,5-dione, Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), 1,4-diaza-2,5-dioxo-3-isobutyl bicycle (4.3.0) nonane, N-Methyl-2-Propyl-5-Butylpiperidine, and Phthalic acid. It was found that retention time was nearby (Fig.1; Table 3, 4).

The compounds that we found including antifungal activities such as oosprein (Nagaoka *et al.*, 2004), imidazole (Grimmrtt, 1997), pharmacological activities such as ergotamine (Tfelt-Hansen *et al*., 2000), 1,2,4-Triazolidine-3,5-dione and 1, 3, 4-Thiadiazole derivatives showed board spectrum including antimicrobial activity, anticonvulsant activity, anti-inflammatory activity (Mishra *et al*., 2011). Similarly with study the red pigment synthesized by the filamentous fungi *Isaria farinose* under submerged culture conditions.

Structural elucidation of the pigment using gas chromatography-mass spectrometry. Fourier transform infrared contains an anthraquinone-related compound (Velmurugan *et al*., 2010). Whereas, extraction the red pigment from *Monascus purpureus* and used it for wool dyeing (Santis *et al*., 2005), and assessed the dyeing potential of red pigment of unknown structure to be produced about *I. farinose* (Nagia and El-Mokamedy, 2007). Moreover, numerous studies indicated that pigment production in submerged culture is affected by various environmental factors, particularly the pH of the medium, temperature, agitation, and carbon and nitrogen sources (Carels and Shephered, 1997). The main groups of pigments and their leucofors include simple benzoquinones, terphenylquinones, pulvinic acids, and derived products, anthraquinones, terpenoid quinines, benzotropolones, compounds of fatty acid origin and nitrogen-containing pigments (betalains and other alkaloids)

(Velisek and Cejpek, 2011). Another research, reported that *Cordyceps pseudomilitaris* BCC 1620 was found to produce two interesting metabolites groups namely ten bioxanthracenes (ES242s) and two alkenoic acids (CP-A,B) [3]. Analysis of 10 free fatty acids namely lauric acid, myristic acid, pentadecanoic acid, palmitoleic acid, palmitic acid, linoleic acid, oleic acid stearic acid, docosanoic acid and lignoceric acid and four free sterols including ergosterol, cholesterol, campesterol and β-sitosterol in natural (wild) *Cordyceps sinensis*, *Cordyceps liangshanensis* and *Cordyceps gunnii*, as well as cultured *C. sinensis* and *C. militaris* were first determined using pressurized liquid extraction (PLE), trimethylsilyl (TMS) derivatization and GC-MS analysis. The results showed that palmitic acid, linoeic acid, oleic acid, stearic acid and ergosterol are main components in natural and cultured *Cordyceps* (Yang et al., 2009). In this study, we found that culturing *C. pseudomilitaris* on PDA and MCM medium showed no difference growth. But, culturing this fungus in MCM broth showed more growth than PDB, in shaking condition. Pigment analysis by GC-MS found more compounds in shaking pigments than static pigments. However, eight compounds were showed in both conditions.



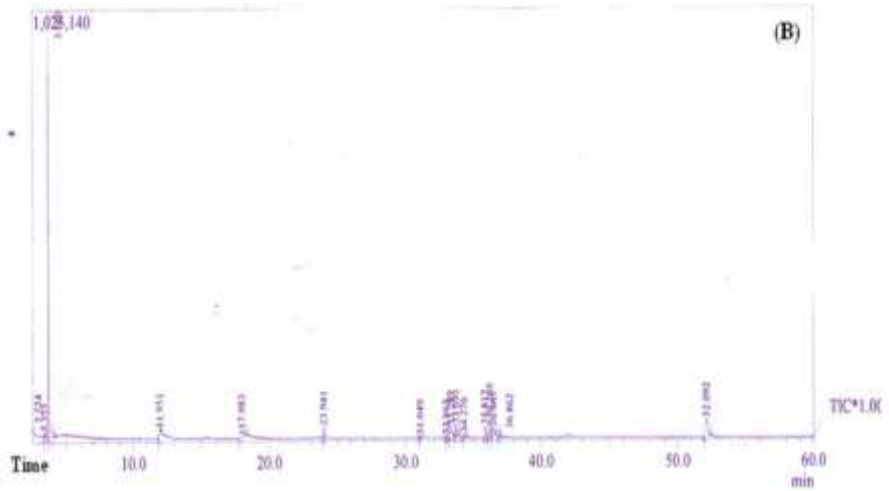


Fig. 1. GC-MS chromatogram of pigments of *Cordyceps pseudomilitaris*
A: shaking condition B: static condition

Table 1. Mycelium growth of *Cordyceps pseudomilitaris* BCC31665 on potato dextrose agar (PDA) and mushroom complete media (MCM)

Media	Colony diameter (cm.)		
	Day after inoculation		
	7 days	14 days	21 days
PDA	1.22±0.08	2.24±0.09	2.72±0.08
MCM	1.30±0.07	2.36±0.05	2.76±0.05
F-test	ns	ns	ns

Table 2. Mycelium dry weight of *Cordyceps pseudomilitaris* BCC31665 culture in potato dextrose broth (PDB) and mushroom complete media broth (MCM broth) after 21 days

Media	Mycelium dry weight (g)	
	Static	Shake
PDB	0.108±0.01	0.166±0.01
MCM broth	0.108±0.01	0.234±0.05
F-test	ns	ns

Table 3. Chemical composition of *Cordyceps pseudomilitaris* pigments that culture in shaking condition by Gas Chromatography – Mass Spectrometry (GC-MS)

Peaks	Retention time (min.)	Compound	%Area	%Similarity Index
1	2.425	1,3-Dioxolane	0.40	79
2	11.681	Mevalonic lactone	19.32	91
3	19.373	5-Undecene	0.27	83
4	23.893	Phenol	3.55	94
5	26.742	1-Hexadecene	1.00	90
6	30.858	2-Undecene	1.42	73
7	31.083	1,1,3-Trimethyl-3-phenylindan	0.66	86
8	32.133	Glycyl-L-proline	3.16	88
9	32.993	1,2,4-Triazolidine-3,5-dione	0.52	88
10	33.208	1-Nonadecene	0.87	91
11	33.649	Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	6.24	82
12	34.242	2-Decene,3-methyl-	2.32	72
13	35.798	5-Nitroso-2,4,6-triaminopyrimidine	7.10	75
14	36.171	1,4-diaza-2,5-dioxo-3-isobutyl bicycle (4.3.0) nonane	11.76	88
15	36.363	N-Methyl-2-Propyl-5-	5.99	77
16	36.426	Butylpiperidine	3.43	72
17	36.889	N-Methyl-N-trifluoroacetyl-2-octylamine	2.57	90
18	37.427	Phthalic acid	0.39	85
19	41.75	1-Nonadecene	9.86	86
20	47.916	3,6-Diisobutyl-2,5-piperazined	2.31	83
21	50.382	Ergotamine-GC	4.58	81
22	64.968	Dihydroergocristine	12.28	93
		1,2-Benzenedicarboxylic acid, diisooctyl ester		

Table 4. Chemical composition of *Cordyceps pseudomilitaris* pigments that culture in static condition by Gas Chromatography – Mass Spectrometry (GC-MS)

Peaks	Retention time (min.)	Compound	%Area	%Similarity Index
1	2.063	Acetone	0.84	94
2	2.324	Alanine	2.37	93
3	3.355	2-Pentanone	0.84	88
4	3.660	Diacetone alcohol	45.53	98
5	11.951	Mevalonic lactone	2.16	82
6	17.983	Crotonic anhydride	7.17	85
7	23.941	Phenol	2.37	84
8	31.049	1,1,3-Trimethyl-3-phenylindan	0.41	74
9	32.963	1,2,4-Triazolidine-3,5-dione	0.41	91
10	33.289	Imidazole-1-D	6.39	84
11	33.691	Pyrrolo (1,2-a) pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	4.08	79
12	34.276	Cyclo-(Pro-Leu)	1.42	82
13	35.817	Cyclo-(Pro-Leu)	3.30	84
14	36.205	1,4-diaza-2,5-dioxo-3-isobutyl bicycle (4.3.0) nonane	5.35	83
15	36.465	N-Methyl-2-Propyl-5-	4.07	70
16	36.862	Butylpiperidine	1.80	81
17	52.092	Phthalic acid Oosporein	11.47	81

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