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Optimization of Pulsed UV Light Irradiation for the Production of Vitamin D_2, Bioactive Metabolites and Antioxidant Activity of *Cordyceps militaris* Mycelia

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Abstract. Pulsed UV light (PUV) delivers intense pulses in a very short time in comparison with continuous UV light. Hence, this study is aimed to discover the optimum dose of PUV light irradiation to produce vitamin D_2, bioactive metabolites and antioxidant activity of *Cordyceps militaris* cultured mycelia. Forty grams of cultured mycelia was put at a distance of 1.25 inches from the lamp and irradiated by 3, 6 and 9 PUV light (broadband energy = 4.208 J/cm^2 per pulse) prior to the evaluation of their adenosine & cordycepin, polysaccharides and antioxidant activity screened by three complementary test systems namely DPPH free radical scavenging activity, chelating ability on ferrous ions and reducing power. Vitamin D_2 of cultured mycelia reached its optimum concentration by 3 pulses irradiation with 0.61±0.02 mg vitamin D_2/g dry weight mycelia. Three pulses irradiation also yielded an optimum concentration of adenosine, a moderate level of cordycepin and relatively high content of polysaccharides of cultured mycelia, given by 2.31±0.02 mg/g, 0.16±0.02 mg/g and 47.03±0.51 mg/g, respectively. Among treatments, mycelia irradiated by 3 PUV light exhibited the optimum antioxidant properties shown by their EC_{50} values. This result suggested that 3 PUV light irradiation with its low energy is the optimum treatment for the production of vitamin D_2, with minor effects on adenosine & cordycepin, polysaccharides and antioxidant activity of *Cordyceps militaris* mycelia.

Keywords: *Cordyceps militaris*, Mycelia, PUV light, Vitamin D_2, Adenosine, Cordycepin, Polysaccharides, Antioxidant activity

1. Introduction

Mushroom is available abundantly in nature and seems to possess the ability to fulfil the highly increased demands on healthy food. Interestingly, mushroom is acknowledged as the only vegan source of vitamin D due to its high content of ergosterol which will be converted to vitamin D_2 (ergocalciferol) when exposed to UV light [1]. Among various mushrooms, *Cordyceps militaris* (L.) Link has been identified to contain beneficial biological activities, for instance: sperm production enhancer [2], longevity promoter, hypoglycemic, anti-tumor, anti-metastatic, hypolipidemic, and antioxidant agent [3]. Besides, *C. militaris*, likely the other species of mushroom, contains high amount of ergosterol, the precursor of vitamin D_2. Since its scarcity in nature, cultivation of *C. militaris* mycelia in submerged culture is currently a significant issue because it yields a great quantity of bioactive compounds in a compact space and shorter time with less chance of contamination in comparison with fruiting bodies cultivation [4].

Pulsed UV (PUV) light enhances vitamin D_2 content of mushroom with intense pulses in short time of irradiation. Thus, physical appearance of mushroom is not affected by discoloration [5]. The study on *C. militaris* mycelia exposed to PUV light is firstly presented in this paper. This research is mainly aimed to investigate the optimum dose of PUV light irradiation on cultured mycelia of *C. militaris* for the production of vitamin D_2, adenosine & cordycepin, polysaccharides and antioxidant activity.

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2. Material and Methods

2.1. *C. militaris* Pre-Inoculum Preparation & Mycelia Cultivation

Mushrooms were initially grown on PDA medium. The mycelia of *C. militaris* were transferred to the seed culture medium by punching out about 5 mm^2 of agar. The seed culture was grown in a 500 ml shake flask containing 200 ml of liquid medium and incubated at 25°C in a rotary shaker (120 rpm) for 5 days. The seed culture liquid medium was composed of: glucose, 20 g/l; yeast extract, 5 g/l; KH_2PO_4, 0.5 g/l; $K_2HPO_4 \cdot 3H_2O$, 0.5 g/l and $MgSO4 \cdot 7H_2O$, 0.5 g/l [6].

2.2. PUV Light Treatment on *C. militaris* Mycelia

Mycelia were harvested by cloth filter filtration and exposed to PUV light (Xenon RC-847 and a LH-810 lamp housing model). A 40 g of mycelia was flattened with 1 mm thickness for each time of irradiation on 120 cm^2 surface area. Three, 6 and 9 pulses were applied and non-irradiated mycelia was the control group, with 1.25" distance of lamp house and sample chamber [5,7]. The broadband energy generated was 4.208 J/cm^2 per pulse. Afterwards, mycelia were freeze-dried and ground into powder (Retsch Ultracentrifugal Mill and Sieving Machine Haan, Germany) to obtain fine powder (40 mesh) for further analysis.

2.3. Ergosterol & Vitamin D_2 Analysis

Using the method of Tung *et al*. [8], 3 g of sample was extracted with 12 ml of DMSO and sonicated (Delta, Ultrasonic Cleaner). A 6 ml of 1:1 MeOH/distilled H_2O was added prior to the addition of 12 ml of N-hexane. Meanwhile, the steps of adding N-hexane until supernatant obtained were repeated. Supernatant was evaporated and followed by MeOH addition. The solution was filtered before the aliquot of 20 µl of pre-prepared mixed standard samples were injected to the HPLC system. The HPLC system consisted of a Hitachi L-2130 pump, a 20 µl sample loop, Hitachi L-2400 UV detector and a LiChrospher 100 RP-18 column (4.6 x 250 mm, 5 µm, Merck), a 20 µl sample loop, under 30°C temperature and 254 nm UV detection. The mobile phase was 95% MeOH/H_2O at a flow rate of 1 ml/min. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.4. Adenosine & Cordycepin Analysis

Following the procedures of Huang *et al*. [9], 5 g of sample was mixed with 100 ml distilled H_2O and sonicated. Collected supernatant volume was precisely measured prior to a 0.45 µm PVDF filter (Millipore) filtration and HPLC evaluation.

The HPLC system and column used was the same as for the vitamin D_2 assay. The mobile phase was 85% 0.02M KH_2PO_4/MeOH at a flow rate of 1.2 ml/minutes and UV detection at 260 nm. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.5. Polysaccharides Analysis

A 0.1 gram of sample powder was refluxed with 2 ml RO H_2O. The homogenate was centrifuged and the precipitate was extracted with 2 ml of 100°C H_2O. The supernatant was dialyzed using Cellu Sep T2 tubular membrane ((MWCO):6,000-8,000, Membrane Filtration Products, Inc., Seguin. TX. USA) for 24h. The polysaccharide content was determined by the phenol-sulfuric acid assay [10]. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.6. Antioxidant Activity

2.6.1. Preparation of Extracts

A 10 g of sample was mixed with 100 ml of 95% ethanol and filtered through Whatman No. 1 filter paper. This step was repeated twice and the extract was evaporated at 40 °C to dryness. The dried extract was used directly for analysis of antioxidant components or redissolved in ethanol to a concentration of 50 mg/ml and stored at 4 °C for further uses.

2.6.1.1. Scavenging Activity on DPPH (1,1-diphenyl-2-picryl hydrazyl) Free-radicals

Briefly, 0.3 ml portion of 0.5 mM DPPH ethanol solution was mixed with extracts [11]. A 70% EtOH was used as control and the solution without extract or BHA was used as a sample blank. The mixture was shaken vigorously and left to stand for 30 min in the dark room. The reduction of the DPPH radical was determined by measuring the absorption at 517 nm and calculated using the following equation:

$$\text{Radical Scavenging Activity (RSA) (\%)} = \frac{\text{Ac-(As-Asb)}}{\text{Ac}} \times 100\%$$

where Ac, As and Asb were the absorbance at 517 nm of the control, samples or BHA with DPPH treatment and samples or BHA blank (no extracts or BHA were added). The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.6.1.2. Ferrous Ions Chelating Activity

A 1 ml of extract was mixed with 3.7 ml MeOH and 0.1 ml of 2 mM $FeCl_2.4H_2O$ and 0.2 ml of 5 mM ferrozine [12]. The absorbance at 562 nm was determined and chelating ability on ferrous ions was calculated as the following equation:

$$\text{Inhibition (\%)} = 100\% - \left(\frac{\text{(As-Asc)}}{\text{Ac}} \times 100\%\right)$$

where As, Asc and Ac are the absorbance at 562 nm of the sample, sample color and control. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.6.1.3. Reducing Power

The Fe^{3+} reducing power analysis was determined by a method described by Bao *et al*.[11]. Reaction mixtures contained 0.5 ml of 1% potassium ferricyanide, 0.5 ml of extract and 0.5 ml of 0.2 M sodium phosphate buffer (pH 6.6). The mixture was incubated at 50ºC for 20 mins and subsequently 0.5 ml of 10% TCA (w/v) was added followed by the addition of 1.4 ml of distilled H_2O and 0.1 ml of 0.1% of ferric chloride. The absorbance of the mixtures was measured at 700 nm against a control. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations (SD).

2.6.4. EC_{50} Value Calculation

EC_{50} value (mg extract/ml) is the effective concentration at which the absorbance was 0.5 for reducing power; DPPH radicals were scavenged by 50%; and ferrous ions were chelated by 50%, respectively. EC_{50} value was obtained by interpolation from linear regression analysis.

2.7. Statistical Analysis

Statistical analysis was evaluated by Statistical Analysis System (SAS) software package. All pooled data represented means ± standard deviations (SD). Analysis of variance was accomplished by ANOVA procedures. Significant differences between means were determined by Duncan's multiple-range test at a level of $P<0.05$.

3. Results and Discussion

The idea of applying PUV light irradiation on mushroom surface has been a breakthrough to encounter vitamin D deficiency problem [5]. However, irradiation intensity needs attention to be paid. This study showed that the content of vitamin D_2 in irradiated mycelia of *C. militaris* achieved its optimum concentration by 3 pulses irradiation. A dramatic rising was shown from control groups which only contained 0.02±<0.01 mg/g of vitamin D_2 to 0.61±0.02 mg/g vitamin D_2 with 3 PUV light irradiation (Table 1.). This is in a positive agreement with the findings of Kalaras *et al*. [7] who reported that after exposure to 3 PUV light, the content of vitamin D_2 on *A. bisporus* increased from 0.005 µg/g DW to 12.6 µg/g DW, which is equivalent to 518% Daily Value/serving. Meanwhile, from ergosterol standpoint, as the number of pulses increased, ergosterol content in mycelia gradually decreased significantly. PUV light generated 4.208 J/cm^2 per pulse energy, which was available for vitamin D_2 synthesis from ergosterol conversion [7]. However, with subsequent 6 and 9 PUV light irradiation, vitamin D_2 concentration declined to 0.45±0.03 mg/g and 0.42±0.02 mg/g, respectively, without any significant differences within the latter treatments. The discrepancy existed presumably because despite the conversion of ergosterol to vitamin D_2 was dependent on radiation dose [13], ergosterol contents and its conversion rate to vitamin D_2 in different types of mushroom were varied [14]. Moreover, ergosterol is not a limiting factor, but the thickness of mushrooms, their geometry and pigments are likely factors affecting the yield of vitamin D_2 [5]. Moreover, UV light has limitation for penetration capacity in food materials depending upon their optical properties, in which UV light penetration capacity reduces as absorption coefficient increases. Opaque liquid materials, such as milk

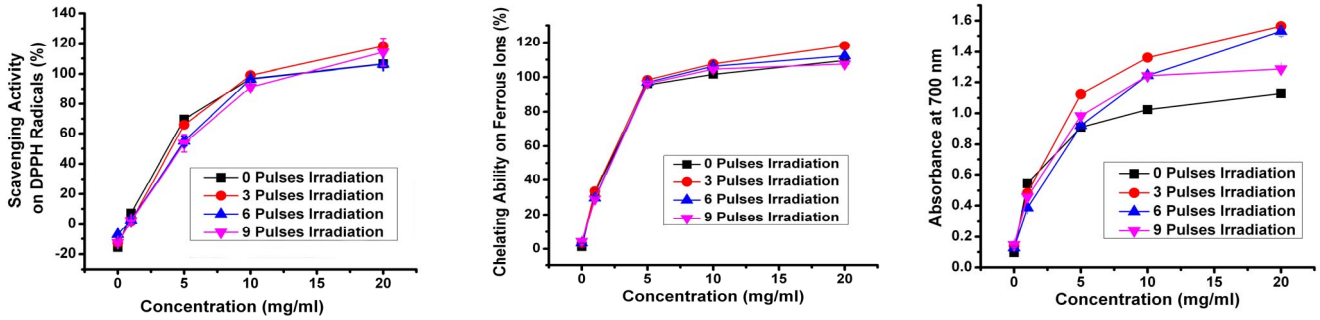
and dark syrup had been known to have high absorption coefficient [15]. Since mycelia were opaque, UV light might not penetrate well which consequently might affect the ergosterol conversion.

Table 1: Effect of PUV light irradiation on the contents of ergosterol, vitamin D_2, adenosine, cordycepin and polysaccharides in *C. militaris* mycelia.

Compounds	Contents (mg/g)			
	0 PUV[a]	3 PUV	6 PUV	9 PUV
Ergosterol	3.45±0.14B[b]	4.56±0.14A	2.63±0.13C	1.85±0.12D
Vitamin D_2	0.02±<0.01C	0.61±0.02A	0.45±0.02B	0.42±0.02B
Adenosine	1.30±0.05D	2.31±0.02A	2.08±0.14B	1.81±0.05C
Cordycepin	0.25±0.01A	0.16±0.02B	0.16±<0.01B	0.01±<0.01C
Polysaccharides	49.28±0.44A	47.03±0.51B	46.59±0.20B	44.03±0.54C

[a]Pulse energy : 4.208J/cm^2/pulse, [b]Means with different letter within a row are significantly different (P<0.05). Each value is expressed as mean ± SD (n=3)

Table 1. represented adenosine content of cultured mycelia obtained its optimum concentration by 3 pulses irradiation with 2.31±0.02 mg adenosine/g mycelia's dry weight. In comparison with control, the concentration of adenosine was enhanced rapidly by 77% increment. However, a slight but significantly different reduction of adenosine level occurred as the number of pulses increased to 6 and 9, with 2.08±0.14 mg/g and 1.81±0.05 mg/g of adenosine concentration, respectively. Whilst, it is also revealed that cordycepin concentration of cultured mycelia significantly decreased as higher number of pulses released. It is shown by 36% reduction from cordycepin concentration of control to 3 pulses irradiated cultured mycelia, which further remained stable by 6 pulses irradiation and significantly decreased to 0.01±<0.01 mg/g by 9 pulses irradiation. Nevertheless, even though this reduction seemed to be influenced by the exposure to PUV light, the content of cordycepin still performed a relatively moderate level at 0.16±0.02 mg/g, and it was surprisingly favorable since cordycepin is present in mycelia grown in solid substrate but is virtually absent in mycelia grown in liquid culture [16].



(a) (b) (c)

Fig 1. Antioxidant Activity of *C. militaris* cultured mycelia irradiated by 0, 3, 6 and 9 PUV light. Scavenging Activity on DPPH Radicals (a), Chelating Ability on Ferrous Ions (b), Reducing Power (c). Each value is expressed as mean ± SD (n=3).

Polysaccharide is abundantly found in *Cordyceps* species. The content of polysaccharides may range between 3% -8% of total weight and usually comes from the fruiting body and mycelia of solid and liquid fermentation submerged culture [17]. Table 1. displayed the concentration of polysaccharides in cultured mycelia was not much affected by high energy of PUV light irradiation. A gradual decrement appeared with significant decline by 4.5% as 3 pulses of irradiation applied to cultured mycelia, from 49.28±0.62 mg/g to 47.03±0.72 mg/g. However, with 6 pulses irradiation, the content of polysaccharides in cultured mycelia slightly decreased but was not significantly different. Subsequently, it dropped significantly to 44.03±0.77 mg/g as 9 pulses irradiation applied. Overall, the concentration of polysaccharides was still considered as high. This result is in a good agreement with the findings of Wong *et al*. [18], who reported that *Cordyceps* polysaccharide has high resistance to UV irradiation when applied to human skin cell.

Result demonstrated that 3 pulses irradiated mycelia showed the highest antioxidant activity in all the three assays. At 10-20 mg/ml extract concentration (Fig 1.), 3 pulses irradiated mycelia displayed the highest scavenging activity on DPPH Radicals, followed by 6 and 9 pulses irradiated mycelia with significant differences, given by 99.03±0.79%, 96.52±0.41% and 91.27±0.46% DPPH scavenging activity

for each treatments with 10 mg/ml extract concentration, respectively and 118.18±0.39%, 106.25±0.55% and 114.18±9.23% for each treatments with 20 mg/ml extract concentration, respectively. Concomitantly, 3 pulses irradiated mycelia performed optimal chelating ability on ferrous ions and reducing power. Antioxidant activity is closely related to cordycepin and polysaccharides concentrations [19]. Since cultured mycelia exposed to 3 pulses irradiation contained fairly sufficient amount of cordycepin and relatively high concentration of polysaccharides, an increasing rate of scavenging activity was possibly achieved, respectively.

Table 2. EC_{50} values obtained in the antioxidant assays of *C. militaris* mycelia irradiated by PUV light.

Compounds	EC_{50} Value (mg extract/ml)			
	0 PUV[a]	3 PUV	6 PUV	9 PUV
Reducing Power	0.44±<0.01D[b]	1.13±<0.01C	1.82±0.01A	1.30±<0.01B
DPPH Scavenging Activity	3.75±0.01B	4.01±0.02B	4.63± 0.22A	4.78±0.46A
Ferrous Ions Chelating Ability	2.10±0.09B	2.01±0.03B	2.21±0.02A	2.01±0.03B

[a]Pulse energy : 4.208J/cm^2/pulse, [b]Means with different letter within a row are significantly different (P<0.05).
Each value is expressed as mean ± SD (n=3).

Carried out in the EC_{50} value evaluation (Table 2.), the antioxidant efficiency was shown in an inverse correlation with antioxidant activity. Among irradiation treatments, 3 pulses irradiation was the most efficient treatment (lower EC_{50} values), concerning antioxidant activity. However, compared to control, 3 pulses irradiation gave slightly lower antioxidant properties (higher EC_{50} values). This loss could be attributed to the thermal damage caused by the use of high fluencies. It has been known that although the antioxidant action can be increased by other substances, for instance tocopherols and β-carotene, DPPH radical scavenging activity of mushroom was discovered to have a positive correlation with gallic acid, pyrogallol, homogentisic acid, protocatechuic acid, catechin, caffeic acid, ferulic acid, naringin, myricetin, quarcetin [20]. Therefore, another investigation is conducted to study the bioactivity of phenolics and flavonoids in *C. militaris* cultured mycelia irradiated by PUV light, considering that phenolics may be correlated to their chelating metal ability, lipoxygenase inhibition and free radicals scavenging activity, while flavonoids are able to act as free radical scavengers and terminate the radical chain reactions that occur during triglycerides oxidation [21].

The use of PUV light irradiation on *C. militaris* cultured mycelia is effective to enhance the production of vitamin D_2. A dose of 3 PUV light irradiation is suggested since it increased the concentration of vitamin D_2 significantly without any major deleterious effects on adenosine & cordycepin concentration, polysaccharides contents and antioxidant properties of *C. militaris* mycelia. In addition, 3 PUV light irradiation would be a reasonable treatment in terms of ease of implementation in commercial setting.

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