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Effect of light-emitting diodes on the production of cordycepin, mannitol and adenosine in solid-state fermented rice by *Cordyceps militaris*

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ABSTRACT

The effects of different light-emitting diode (LED) conditions including different LED wavelengths and LED wavelength combinations on the production of bioactive compounds of *Cordyceps militaris* cultivated on brown rice were investigated. The results of our study showed that the optimal illumination times for biomass (0.49 g/g dw, dry weight), cordycepin (3′-deoxyadenosine) (3.97 m g/g dw), mannitol (21.3 mg/g dw) and adenosine (0.95 m g/g dw) production were 12, 12, 12 and 8 h/day, respectively, by fluorescent lamps. Among the three different LED wavelengths tested, the greatest effect of wavelength for biomass (0.38 g/g dw), cordycepin (2.89 m g/g dw), mannitol (23.6 mg/g dw) and adenosine (0.76 m g/g dw) production were red light (619–626 nm), green light (526–531 nm), red light and blue light (467–472 nm), respectively. Among the ten LED wavelength combinations, the greatest effect of wavelength ratios for specific productivity of cordycepin (30.0), mannitol (86.5) and adenosine (5.5) were 3R:3B,2R:4B and 2R:4B, respectively. Our findings suggest that the combination of wavelengths had the greatest impact on the bioactive compounds production of *C. militaris*.

1. Introduction

An entomopathogenic species, *Cordyceps militaris* (L.:Fr.) Link (Clavicipitaceae, Ascomycetes) is one of the traditional Chinese medicinal mushrooms, It has been reported that the extract of *C. militaris* fruiting bodies showed angiogenesis (Yoo et al., 2004), anti-inflammatory (Won and Park, 2005; Rao et al., 2010), anti-asthma (Hsu et al., 2008), anti-tumor (Rao et al., 2010) and anti-diabetic (Cheng et al., 2012). *C. militaris* contain some noticeable bioactive compounds such as cordycepin (3'-deoxyadenosine), mannitol, and adenosine. Cordycepin is reported to inhibit lipopolysaccharide-induced inflammation (Kim et al., 2006; Tuli et al., 2013), prevent hyperlipidemia (Guo et al., 2010), induce apoptosis in human neuroblastoma and melanoma cells (Baik et al., 2012), induce apoptosis and inhibit the proliferation of cancer cells (Tian et al., 2015), exhibit anti-metastatic activity, and inhibit platelet aggregation (Tuli et al., 2013). Mannitol is used to treat acute stroke (Bereczki et al., 2007) and as a protective and therapeutic agent during neurological or renal failure (Jain et al., 2004). Adenosine has a number of biological activities which allow it to be used as a cardioprotective and therapeutic agent for chronic heart failure (Kitakaze and Hori, 2000), a neuromodulator, and a homeostatic modulator in the central nervous system (Gomes et al., 2011), and it has been to shown to strongly inhibit HIV-1 (Jiang et al., 2011).

Generally, there are three stages in the cultivation of *C. militaris* fruiting bodies during two months of solid-state fermentation by grain substrates (Liang et al., 2014); the mycelium colonization stage, the primordial initiation stage, and finally, the fruiting stage. Light is a necessary factor after the primordial initiation stage of *C. militaris* cultivation. Fluorescent lamps are commonly used currently in the industrial cultivation of *C. militaris* (Wu et al., 2016). However, these conditions take a long time and induce high energy costs to cultivate *C. militaris* fruiting bodies. The Japanese food "natto (*Bacillus*-fermented soybean)" and the Chinese traditional food "anka (*Monascus*-fermented rice)" were produced by solid state fermentation. In recent years, mushroom fermented grain products were studied (Liang et al., 2009a, b, 2013). *C.*

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militaris also can be inoculated into grains, and its fermented grain product will be a novel functional food, providing beneficial effects.

In recent years, there have been advances in light-emitting diode (LED) technology development, and many studies on the effects of LED on the growth of plants (Hogewoning et al., 2010; Hernández and Kubota, 2012; Olle and Viršilė, 2013) and algae for horticultural applications (Mitchell, 2015). To the best of our knowledge, there were few reports on the application of LED light in the cultivation of *C. militaris* (Dong et al., 2013; Yi et al., 2014). Particularly, limited information about the effect of different LED light combinations on the culturing of *C. militaris* is available. In this study, LED light of different wavelengths and their combinations were used during the culture process to investigate the effects on the mycelial growth and contents of bioactive compounds of *C. militaris* when cultivated on brown rice.

2. Materials and methods

2.1. Reagents and standards

Potato dextrose agar (PDA) and yeast extract were purchased from Himedia Laboratories (Mumbai, India, USA). Glucose, cordycepin, mannitol, adenosine and ergosterol were obtained from Sigma-Aldrich Co. (St. Louis, MO). KH_2PO_4, K_2HPO_4, and $MgSO_4{\bullet}7H_2O$ were purchased from Kanto Chemical Co. (Tokyo, Japan). Hexane, methanol and acetonitrile were purchased from Macron Chemicals (Phillipsburg, NJ, USA). LED lights were purchased from Everlight Electronics Co., Ltd. (New Taipei City, Taiwan) and incubator includes fluorescent lamps were purchased from Yih-Der Co., Ltd. (New Taipei City, Taiwan).

2.2. Microorganism

The strain of *C. militaris* 101 was kindly provided by Hsing-Tai Biotechnology Co. Ltd. (Changhua, Taiwan). Stock cultures were maintained on potato dextrose agar (PDA) slants. Slants were incubated at 25 °C for 14 days and stored at 4 °C for use as subcultures every two months.

2.3. Liquid spawn preparation

C. militaris was initially grown on PDA medium at 20 °C and the mycelium harvested after 14 days. Five mycelial agar discs (5 mm × 5 mm) were obtained using a sterilized knife and were transferred to 250-mL flasks containing 100 mL of GY medium (glucose, 20 g/L; yeast extract, 5 g/L; $MgSO_4{\cdot}7H_2O$, 0.5 g/L; K_2HPO_4, 0.5 g/L; KH_2PO_4, 0.5 g/L) under laminar flow. Flasks were shaken at 100 rpm at 20 °C for 3 days on an orbital shaker. Liquid products of the mycelial culture were used directly as liquid spawn.

2.4. Grain substrate preparation, inoculation, and incubation

The brown rice (purchased from I-Mei Foods, Co., Ltd., Taipei, Taiwan) substrates were prepared by filling wide-neck glass bottles with 10 g of rice grains and 15 mL of water and autoclaving at 121 °C for 15 min. Substrates were cooled to room temperature and inoculated with 3 mL of liquid spawn. Inoculated substrates were kept in a spawn running incubator room at 20 °C under dark conditions for 10 days. Once the substrates were covered by white mycelium, they were moved to a low-temperature room (4 °C) for 24 h. Afterwards, the substrates were transferred to light conditions at 20 °C and subjected to different LED light treatments for 15 days.

2.5. Illumination times

To investigate the effects of different illumination time on the production of bioactive compounds of *C. militaris*, the substrates in the light culture incubator room were illuminated at a light intensity of 1400 ± 250 lux for 4, 8, 12, 16, and 20 h/day by fluorescent lamps and under dark condition as a control. Triplicate substrate cultures were exposed to each light treatment for 15 days.

2.6. Different wavelengths of LEDs

To investigate the effects of different wavelengths of LEDs on the production of bioactive compounds of *C. militaris*, the substrates in the light culture room were exposed to red light LED (619–626 nm), green light LED (526–531 nm) and blue light LED (467–472 nm) for 12 h/day. The control was illuminated by fluorescent lamps for 12 h/day. Triplicate substrate cultures were exposed to each wavelength of LEDs treatment for 15 days.

2.7. Combination of different ratios of wavelength of LEDs

Two combinations of different ratios of wavelengths of LEDs were applied for 12 h/day. These two types were (1) a combination of red (R) and blue (B) LED, at ratios of 5B:1R, 4B:2R, 3B:3R, 2B:4R, 1B:5R, and (2) a combination of blue (B) and green (G) LED, at ratios of 5B:1G, 4B:2G, 3B:3G, 2B:4G, 1B:5G. The control was illuminated by fluorescent lamps for 12 h/day. Triplicate substrate cultures were exposed to each combination of LEDs treatment for 15 days.

2.8. Sample preparation for extraction

The harvested fermented grain substrates under different illumination treatments were dried at 60 °C in an oven (Yih-Der LM-570RD, New Taipei City, Taiwan) to a constant weight (The moisture content of the dried samples was between 6.86% and 7.58%), before being ground into powder with a grinder (Retsch, Haan, Germany). The sample powders were stored in an opaque desiccator and kept in 25 °C for at most 1 month until the extract preparation.

2.9. Analysis

2.9.1. Validation of analytical methods

Limit of determination (LOD) and limit of quantification (LOQ) were determined by analyzing 10 blank samples. The LOD and LOQ were determined as the minimum concentration based on 3 and 10 times the standard deviation of the signals from the blank samples, respectively. The precision and recovery were evaluated at two levels for four analytes in quadruplicate. The precision was evaluated by analyzing the coefficient of variation (CV) for intra-day assay on the same day, and inter-day assay were determined after 3 days. The recovery capacities of the methods were assessed by spiking sample powders with two different concentrations of pure standards.

2.9.2. Determination of ergosterol content

Ergosterol, a major fungal sterol, can indicate the level of mycelia in fungi (Seitz et al., 1979). In this study, ergosterol was a measure of *C. militaris* growth in grain substrate. Extraction of ergosterol from fermented grain substrates was conducted according to the method of Matcham et al. (1985) with some modification. One gram of dry fermented grain powder was vortexed with 4 mL of hexane for 90 s, centrifuged at 1310g for 10 min, and the supernatant (hexane phase) transferred into a vial. The residue was further extracted twice with 4 mL of hexane. The