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Medium Composition Effects on Growth Kinetic of *Cordyceps militaris* Cells Using Agar Plate Method

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Abstract: *Cordyceps militaris is one of the well described mushrooms and has been extensively used in Traditional Chinese Complementary Medicine (TCCM) since many centuries. It has been cultivated naturally or in submerged culture using different types of media. Fungal mycelia contain adenosine, cordycepin, and polysaccharides, which are responsible for its biological activities. Cordycepin is the best-known and most potent mushroom-derived substances possessing anticancer, antitumor and immunomodulating activities. This mushroom characterized by very low growth rate when grown in solid agar medium for inoculum preparation during cultivation process. Thus, there was enormous need to increase the growth rate of this type of mushroom on solid medium to reduce the time of inoculum preparation stage. In this study, optimization of agar cultivation medium for rapid cell growth using modified potato dextrose agar (PDA) medium culture supplemented with specific amount of malt extract (ME) together with yeast extract (YE) was investigated. The mycelial growth diameter was monitored during 21 days of cultivation using two series of experiments of different medium supplements: 2, 4, 6 and 8 g of ME and 6 g of ME with 0, 2, 4 and 6 g of YE to the PDA medium. The obtained results clearly demonstrate that the highest mycelial growth diameter of about 7.5 cm was obtained in PDA medium supplemented with 6 g/L ME and 4 g/L. YE.*

Keywords: *Cordyceps militaris, solid culture, mushrooms, mycelium growth, medium optimization*

I. Introduction

Cordyceps is one of the famous medicinal mushrooms from the family of Clavicipitaceae and the order Hypocreales [1-4]. To date, more than 350 species of Cordyceps were discovered all around the world, of which about 120 species have been originated in China [1,5]. *Cordyceps sinensis* (Berk.) Sacc. Is a well-known genus of Cordyceps, and found as fungal parasite in the larvae of Lepidoptera. By infecting caterpillar and devouring the host at the end of autumn, the fruiting body like grass protrudes from the head of the lifeless host in the early summer. For thousands of years, Cordyceps species have been used widely in China and Japan for their valuable therapeutic activities in making general tonics and aphrodisiac. However, the slow growth rate and specific altitude condition of this medicinal mushroom are the main concern for inadequate supplies to meet the market demand. Recently, mycelial fermentation technique and fruiting body as an alternative for steroidgenesis have been used to cultivate Cordyceps species artificially. However, the main mechanism of fungal toxicity is not elucidated clearly [2]. In addition, it was found that *C. sinensis* cultivated artificially demonstrates similar bioactivities as its wild or natural type [6]. Furthermore, it is reported that Cordyceps and its anamorph contain various bioactive ingredients [7], such as adenosine, cordycepin, polysaccharides, and ergosterol with wide therapeutic activities [8.9]. 3-deoxyadenosine or cordycepin as an analogue of the nucleoside adenosine structured by the ribose unit without oxygen in its 3′ position is one of the most significant bioactive ingredients extracted from various mushrooms. Typically, cordycepin is found in the lowest amount with many difficulties in the *C. sinensis.* As opposite, the amount of cordycepin is much higher in *C. militaris*. A report was published which presented that cordycepin is (appealed to be) a significant compound in TCM that is given for different illnesses, such as chronic inflammation and cancer [10]. Furthermore, It has been reported that cordycepin is converted inside the cells into its 5′-mono, di and triphosphates and they hinder the movement of ribose-phosphate pyrophosphokinase and 5-phosphoribosyl-1-pyrophosphate amidotransferase in the de novo purines biosynthesis and/or the nucleic acids synthesis causing the anti-metastatic, anti-tumor and anti-microbial results [11-17]. In addition, the anti-leukemic activity of cordycepin joined together with adenosine deaminase inhibitor and the inhibitory effect of its analogues of 2′, 5′- oligoadenylate on human immunodeficiency virus infection

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have additionally been presented [18,19].

Large scale mushroom cultivation through artificial media has newly been established as a source of a cordycepin substitute because of its limited amount from natural sources. Regarding to submerged cultivation, the two-stage control of dissolved oxygen (DO) in the medium or extra feeding of ammonia to the medium at the correct time was done to improve the production of cordycepin [20,21]. While cordycepin can also be chemically manufactured, the yield is not actual high as well as the difficulties of being a complicated procedure, and furthermore, a huge amount of organic solvents, which are destructive substances for the environment, should be applied in this process [22]. Regarding to artificial cultivation of *C. militaris*, the main challenge is about the growth rate of mushroom cells on the industrial scale preparation by making the cultivation time shorter together with increasing the cell inoculation density. Another similar study was conducted in order to increase the cultivation of *Helvella Crispa* (Scop.) Fr. mycelium on solid-state agar medium [23]. Thus, in order to increase the mushroom growth rate, the effect of using malt extract (ME) as a supplement to classic medium culture contained potato dextrose agar (PDA) and yeast extract (YE) on the growth and cultivation time of *C. militaris* mycelia cells was investigated.

II. Materials and methods

Cordyceps militaris DSMZ 23612 was used throughout this study. This strain was initially obtained from the German microbial and cell culture collection (Braunschweig, Germany). Prior to master cell banking, the strain was activated in a potato dextrose agar (PDA) to ensure the viability of the cells. Agar plates were incubated at 26 ± 1 °C for 21 days followed by keeping in the fridge at 4 °C. Sub-culturing were made from the master cell culture on petri dishes on PDA medium to produce working cell cultures which are further incubated at 26 ± 1 °C. Potato Dextrose Agar medium (PDA), Malt Extract (ME) and Yeast Extract (YE) were purchased from Oxoid Co. (UK)

Preliminary experiments were carried out using PDA medium as control standard medium (Figure 1). Additionally, the growth of C. militaris mycelia cells was monitored through two different series of medium culture, as PDA supplemented with ME or with ME and YE at various concentrations. Results were collected by daily measurement of mycelia cell growth diameter on the petri dishes for 21 days. All data are presented in average with standard deviation obtained from three experiments.

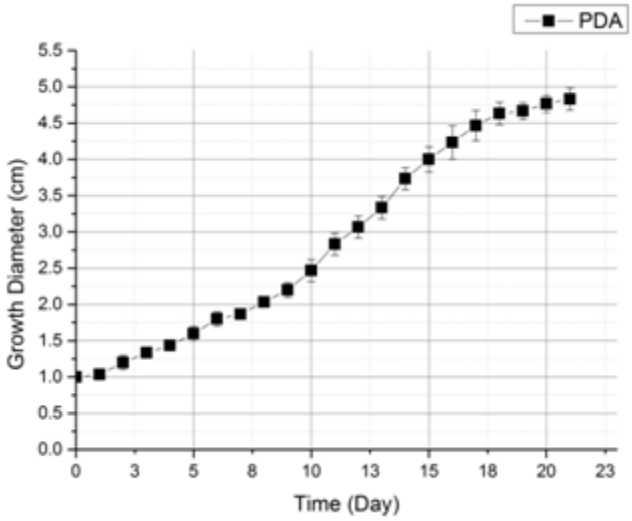

Figure 1. Growth diameter of *Cordyceps militaris* mycelia on the potato dextrose agar (PDA) medium culture

III. Results and discussion

The results of mycelia growth diameter on PDA medium, PDA medium supplemented with different concentrations of ME are presented in figure 2. As shown, all media supplemented with ME supported higher growth of mushroom in all concentrations applied. However, increasing ME concentration more than 6 g/L did not exhibited further increase in growth diameter in mushroom. In initial PDA medium, the growth diameter of mushroom increased gradually and reached 4.8 cm diameter after 21 days (figure 1). In ME supplemented culture of 6 g/L, the mushroom growth reached a maximal diameter of about 6.8 g/L after only 17 days. This indicates that ME not only increase the mushroom growth diameter but also shorten the cultivation time by 5 days.

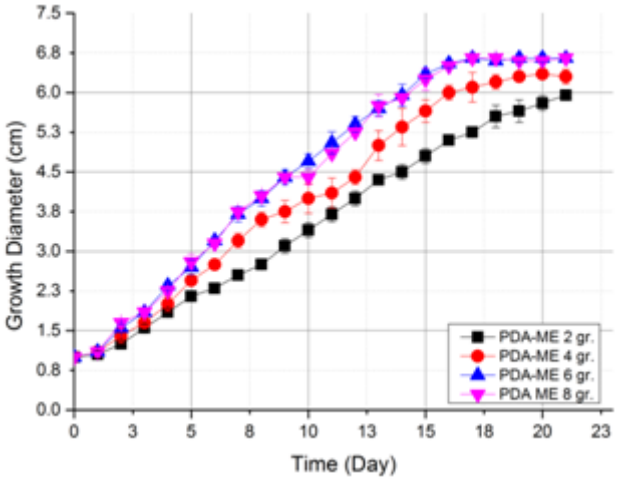

Figure 2. *Cordyceps militaris* mycelia growth diameter on the potato dextrose agar (PDA) medium culture supplemented with 2, 4, 6, and 8 g malt extract (ME)

For further optimization of mycelial growth, yeast extract was supplemented to the growth medium in different concentrations between 0-6 g/L to the best medium chosen in the previous experiment (PDA medium supplemented with 6 g/L ME). As shown on the Figure 3, the highest growth diameter recorded was 7.7 cm for 4g/6g (YE/ME) supplemented culture. Further increase in YE concentration did not show any increase in mycelial growth.

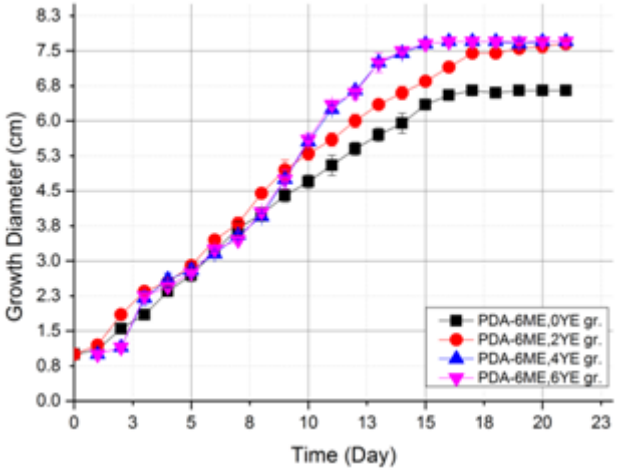

Figure 3. *Cordyceps militaris* mycelia growth diameter on the potato dextrose agar (PDA) medium culture supplemented with 6 g malt extract (ME) and 2, 4 and 6 g of yeast extract (YE)

Potato dextrose agar medium (PDA) is usually considered as the best chosen medium for high fungal vegetative growth and thus it is widely used as standard for mushroom cell inoculum preparation [24]. However, based on the very low growth rate of mushrooms during in vitro cultivation, growth in most cases takes 14-20 days on agar plat before obtaining suitable vegetative growth for next step transfer to vegetative culture. In this study, ME showed positive influence on cell growth. This based on the fact that ME is rich source of carbohydrates mixture which are required to support higher growth of mushroom cells such as maltose, sucrose and other easily assimilated sugars. Furthermore, YE is not only considered as source of nitrogen but also as an excellent source for amino acids, vitamins and other growth factors such as A and B factors [25], which are necessarily for the growth of mushrooms. It was also reported by many authors that, YE supplementation to the fermentation medium has an optimal value and strongly support the production of primary and secondary metabolites [26,27].

IV. Conclusion

The results obtained in this study clearly demonstrate the stimulatory effect of ME and YE on the kinetics of cell growth on agar medium. Thus, addition of both ingredient will resulted on better inoculum preparation in shorter time and thus can reduce the overall process of bioactive metabolites production of

Cordyceps in fermentation system. In addition, this newly obtained medium formulation could be also applied for cultivation of other mushrooms to reduce the time needed for inoculum preparation and vegetative culture growth.

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