International Journal of Innovation Scientific Research and Review
Vol. 03, Issue, 11, pp.1999-2002, November, 2021
Available online at http://www.journalijisr.com
SJIF Impact Factor 4.95

IJISRR

ISSN: 2582-6131

Research Article

THE CULTURE OF CORDYCEPS (Cordyceps militaris) ON SYNTHETIC SUBSTRATES UNDER ARTIFICIAL CONDITIONS

[1,] * Nguyen Ngoc Huy and [2]Phan Ho Anh Phuong

[1]Hue University of Medicine and Pharmacy, Vietnam.
[2]Ton That Tung Junior High School, Vietnam.

Received 20th September 2021; Accepted 21th October 2021; Published online 30th November 2021

ABSTRACT

Currently, Cordyceps militaris has been overexploited in the wild, so it is very scarce. Therefore, the culture of Cordyceps militaris on synthetic substrates under artificial conditions is essential and has been successfully studied. The results showed that the culture of Cordyceps militaris fungus on a synthetic medium of 35g brown rice/box + 60ml mineral solution + 5% dry pupae powder made the mycelium grow fast (after six days of culture, covering the surface of the medium) and the number of fruitbodies is high (average 60 fruitbodies/jar).

Keywords: fruitbodies, culture, Cordyceps militaris, a synthetic medium.

INTRODUCTION

Cordyceps is a fungus that parasitizes the larvae, pupae, or adults of some insects. Up to now, more than 400 species of Cordyceps fungi have been discovered in the genus of *Cordyceps*, but only two species that are *Cordyceps sinensis*and *Cordyceps militaris* have been studied the most because of their high medicinal value. Therefore, Cordyceps has been overexploited in the wild, leading that it is extremely scarce and has prohibitively expensive prices that many people cannot afford to buy and use. Due to the secrecy of technology, there have been very few publications on the culture of *C. militaris*so far. Therefore, the development of studies on the culture of Cordyceps (C. militaris) under artificial conditions aims to be proactive in technology and increase production scale, contributing to reducing product costs. Creating opportunities for everyone to access products and serve health care is very necessary. Therefore, we studied and carried out the culture of Cordyceps militaris mushroom on synthetic substrates under artificial conditions and achieved successful results.

OVERVIEW

In the wild, Cordyceps is commonly found in the summer. The *C. sinensis* is distributed mainly in the high mountains of the Himalayas with an altitude of over 4000meters above sea level such as in Tibet (China) and in some regions of Nepal and Bhutan; The *C. militaris* is found in the lower mountains with the altitude of 2000 - 3000 meters, and it is widely distributed (in China, Japan, Korea and countries in Southeast Asia) [1]. *C. militaris* species has a content of bioactive substances such as cordycepin, mannitol, cordypolysaccharide, superoxide dismutase, amino acids, adenosine, and many other equivalent components or even higher than that of *C. sinensis* species, but easily successfully cultured in the artificial environment [2]. Cordyceps (*C. militaris*) contains a lot of precious medicinal active ingredients, so it is very good for the human body, helping to treat and fortify the immune, digestive, circulatory, nervous, respiratory,and reproductive systems of the body [3].

RESEARCH SUBJECTS AND METHODS

Research object

The Cordyceps species is *Cordyceps militaris* strain provided by Forest Biotechnology Institute.

Research methods

Research overview theory

Review of documents on growth characteristics, morphology, chemical compositions, and uses of Cordyceps:

The genus of Cordyceps has more than 600 different species. However, only two species of *Cordyceps sinensis* and *Cordyceps militaris* have been studiedso far. Since 1964, only *Cordyceps sinensis* has been considered a medicinal herb in pharmacopeia. *Cordyceps sinensis* (also: *Ophiocordyceps sinensis*) is a parasite on insect larvae and is distributed mainly in Tibet, grasslands in Nepal, Bhutan, and northern India, where the altitudes are 3500-5000meters above sea level. *Cordyceps sinensis* has been marketed as a nutritional supplement under FDA control, so the market demand for cordyceps has increased in many countries. In the Vietnamese market, the price of natural Cordyceps is about VND 70,000,000 to VND 120,000,000 for 100 grams of from 600 to 200 pieces.[1] With such a high price, many people cannot access this precious medicinal herb. At the same time, suppliers have increased their exploitation of cordyceps in the wild, depleting the resources of this special medicinal species. Stemming from the needs of the market, in order to meet more widely the demand for using this precious medicinal herb, researchers have

[1] Cordyceps militaris is a entomopathogenic fungus, meaning it parasitizes insects.
[2] Portal of On-plaza Cordyceps Store "Price of Cordyceps"

sought to culture this fungus in artificial conditions. Till the mid-1990s, the artificially cultured *Cordyceps sinensis* has been widely marketed around the world.

Taxonomy of *Cordyceps sinensis*:

- Kingdom - Fungi
- Phylum - Ascomycota
- Class – Ascomycetes
- Order – Hypocreales
- Family – Clavicipataceae
- Genus - Cordyceps
- Species - Cordyceps sinensis

Morphological characteristics of *Cordyceps sinensis*: The fruit body of *C. sinensis* is dark brown to black, usually arising from the head of the larva of the worm *Hepialisarmoricanus*. The body of the larva is yellow or brownish-yellow. When the fruit body matures, it will form spores that easily leave the sporangium and disperse in the wind or fall to the ground. Cordyceps is both an animal and a plant combined with fungi and larvae. In winter, the fungi parasitize the larvae's body and take all the nutrients, causing the larvae to die. Until summer, the fungi will grow to the surface and are called Cordyceps. When Cordyceps is alive, it is clearly seen that it has the shape of a worm, with a small branch in the tail, growing leaves. When dried, it has a fishy smell, while when burned up, it has a fragrant aroma. The “leaf” part is shaped like a finger, about 4-11cm long because the mycelium grows attached to the larva's head. The larva's head as a silkworm is about 3-5 cm long and about 0.3 - 0.8 cm in diameter. The outside is dark yellow or golden brown with about 20-30 rings. The ones near the head are smaller. The head is red-brown. The tail is like a silkworm's tail. There are eight pairs of legs in all, but the four pairs in the middle are the most obvious. Curved rod-shaped stroma grows from the larva, but slightly longer than the larva. The larvae are easy to break with the whole and quite yellowish-white inside. The stroma is quite challenging and the inside is a bit hollow with ivory white color. Wild Cordyceps are harvested in the summer from March to July every year. After harvested, the cordyceps are washed and dried. Cordyceps can use both the larva and the mycelium emerging from the stem. The easiest way to store dried cordyceps is to put them in a sealed plastic bag and refrigerate them at about 4 Celsius. Thus, it can be used for a few months without worrying about its medicinal properties. The chemical analysis shows that in the biomass of cordyceps, there are 17 to 19 different amino acids, D-mannitol, lipids, and many trace elements (Na, K, Ca, Mg, Al, Mn, Cu, Zn, Bo, Fe, etc. in which phosphorus is the highest). More importantly, in the biomass of Cordyceps, there are many substances -6- with biological activities that scientists have been gradually discovered, thanks to the advancement of the chemistry of natural compounds. Many active ingredients in cordyceps have miraculous medicinal value. Among them are cordiceptic acid, cordycepin, adenosine, and hydroxyethyladenosine. The most notable is the functional group HEAA (Hydroxy -EthylAdenosine - Analogs). Cordyceps also contains many vitamins (in 100g of cordyceps, there are 0.12g of vitamin B12, 19 mg of vitamin A, 116.03 mg of vitamin C, in addition to vitamin B2 (riboflavin), vitamin E, vitamin K, etc.). Additionally, there are about 25 - 30% protein, 8% fat, and mannitol sugar. According to Holiday and Cleaver (2004), cordyceps has been used as a “panacea” since 620 AD, during the Tang Dynasty in China (618-907). Countries such as Korea, Japan, Vietnam, etc. have all succeeded in culturing cordyceps on silkworm larvae or other hosts such as the mix of green beans, eggshells, brown rice, and ground silkworm pupae. In 1994, China officially classified cordyceps as a medicine. After that, cordyceps was used a lot when the SARS epidemic appeared in China in 2003. Recently, cordyceps has been shown to be effective in treating cardiovascular, respiratory, liver, and kidney diseases, inhibiting tumor formation, etc. [4]. Experimentally, water and alcohol extracts, both natural and cultured cordyceps, showed antioxidant effects. Inhibiting the oxidizing capacity of linoleic acid, inactivating the substance 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydrogen peroxide, hydroxyl radicals, superoxide anion, metal-capturing activity, polyphenolics, and flavonoids in Cordyceps are anti-oxidants. Research on different types of cancer cells such as lymph node, liver, colon, prostate, and breast, shows that alcohol extract from Cordyceps has an anti-proliferative effect on these cancer cells. Another study showed that cordyceps inhibited colon cancer cell proliferation by inhibiting the degradation of intracellular I-kappa B-alpha and inhibiting NF-Kappa B activity. The hot water extract of Cordyceps sinensis has anti-fatigue and stress effects in ICR mice and Sprague-Dawley rats. The alcohol extract results in inhibiting the proliferation of BALF (Bronchoalveolar lavage fluids) cells activated by lipopolysaccharide (LPS), and inhibiting the production of IL-1 beta, IL-6, IL-8, IL -10, and INF - alpha on BALF. In a model of Sprague-Dawleyrat, inducing liver fibrosis with Dimethyl nitrosamine, orally administered Cordyceps sinensis, the results showed a significant reduction in liver fibrosis because it promotes the degradation of collagen substances such as Hydroxyproline, inhibits metalloproteinase-2 in tissues, type IV and type I collagen.

The study was conducted on recovered SARS patients, divided into two groups:

- Trial group: 16 people (4 men and 12 women); average age 34.3 years old; give 3 grams of Cordyceps per day.
- Control group: 15 people, cared for by modern medicine.
- Results assessed by CT scan of lung and Serum soluble Interleukin-2 Receptor (SIL-2R) test: the trial group improved well on CT scan and reduced SIL-2R concentration, while the control group did not get these results.

The immunomodulatory properties of polysaccharides from *Cordyceps sinensis* were investigated by a peripheral blood smear test. Results: The extract was able to induce the production of tumor necrosis factor-alpha (TNF-alpha), Interleukin (IL)-6, and IL-10. From these studies, Cordyceps is used to treat cough, chronic bronchitis, kidney disease, nocturia, male hypogonadism, anemia, high cholesterol, liver dysfunction, fatigue, tinnitus, weight loss, etc.; To enhance immune system function, to boost athletic performance, to fight premature aging, to improve liver function in people with hepatitis B. Some people use cordyceps as an “adaptogen” to increase fitness and combat fatigue. For the cardiovascular system, Cordyceps is suitable for the heart and blood vessels, regulates heart rate, lowers blood cholesterol, inhibits platelet aggregation, has anti-inflammatory effects, and improves collateral circulation. Myriocin and thermozymocidin (an atypical amino acid) effectively inhibit serine palmitoyltransferase, formed in the early stages of sphingosine biosynthesis [5]. Myriocin is 10-100 times more immunosuppressive than cyclosporine.

Practical research

- Experimental production.
- Test products according to safety standards.
- Build production procedure.

Device

We are using machinery and equipment at the Biology laboratory, Faculty of Basics, University of Medicine and Pharmacy, Hue University: Air conditioner. In addition, we have to buy more: humidifier, hygrometer, led long light, shelf for culture jars.

RESULT

Culture process

After doing an overview of the growth and development of *Cordyceps militaris*, we cultured it according to the following formulas:

Formula 1: 35g of brown rice/jar + 55 ml of mineral solution;
Formula 2: 35g of brown rice/jar + 15% fresh pupae liquid + 55 ml of mineral solution;
Formula 3: 35g of brown rice/jar + 20% fresh pupae liquid+ 55 ml of mineral solution;
Formula 4: 35g of brown rice/jar + 25% fresh pupae liquid + 55 ml of mineral solution;
Formula 5: 35g of brown rice/jar + 5% dry pupae powder + 55 ml of mineral solution;
Formula 6: 35g of brown rice/jar + 7% dry pupae powder + 55 ml of mineral solution;
Formula 7: 35g of brown rice/jar + 9% dry pupae powder + 55 ml of mineral solution.

The time for *C. militaris* to start growing fruit bodies ranges from 20 to 23 days. Statistical analysis results showed no significant difference in the time of initiation of fruit body formation among the formulas.

Fruit bodies in the condition without light and with light have apparent differences in morphological characteristics:

- Fruit bodies in no light are ivory white, and the average diameter is from 0.2 to 0.4 cm.
- Fruiting bodies in light conditions (> 12 hours of light) are orange-yellow, and the average diameter is from 0.2 to 0.3 cm.

Culture results

After culturing in 07 different formulas, (Formula 1 –Formula 7). Each procedure was carried out with 50 culture jars and repeated three times. The conditions in the culture were stable and the same. Giving the same amount for each jar (5% breed).After injection, apply to darken incubation for the mycelium to cover the medium. Next, the jars were transferred to the lighting stage to stimulate the sprouting of the fruit bodies and take care of the fruit bodies with lighting conditions of 1000 Lux, humidity of 85%, and 22Celsius degrees. Monitoring and statistics of fungal growth at the following times are the mycelium system covered the medium jar, the onset of fruit bodies, the number and size of fruit bodies in each jar of the culture formulas. We obtained the following results:


Formula 1


Formula 2


Formula 3


Formula 4


Formula 5


Formula 6


Formula 7

DISCUSSION

We found that when culturing fungi on seven different nutrient media formulations, the growth and development of the mycelium system and the ability to form fruit bodies are different. Those differences are clearly shown in norms such as time for the mycelium to fully cover the environment, time to appear fruit bodies, the number of fruit bodies, and the size of fruit bodies. Specific data is shown in the following table:

Table of the ability to grow, develop and form fruit bodies of strains of *C. militaris* on different media formulations

Formula	Quantity	Characteristics
Formula 1	40 ± 5	Fruitbodies are slender, in medium length, yellow-orange, and white
Formula 2	42 ± 4	Fruitbodies are thin, small, low, and yellow-orange
Formula 3	48 ± 3	Fruitbodiesare large, dark, long, and light orange
Formula 4	57 ± 6	Fruitbodies are small, in medium length, orange, and white
Formula 5	53 ± 3	Fruitbodies are large, long, and orange
Formula 6	56 ± 2	Fruitbodies are moderately large, moderately long, yellow-orange, and white
Formula 7	60 ± 4	Fruitbodiesare medium-large, long, orange and white

Formula 1 obtained the least number of fruit bodies (40 ± 5), while formula 7 obtained the most significant number of fruit bodies (60 ± 4). The speed of the mycelium covering the surface of the medium is from 5 to 10 days. The fastest is in formula 4 (5 days), and the slowest is in formula 1 (10 days). The reason is that the composition and nutritional content in the culture medium of those formulas are different. The source of nutrition is fresh ground silkworm pupae liquid which contains high nutritional content. It is easier to use than dried pupae so that the mycelium can be used directly, leading to faster growth than non-nutritive formulas of silkworm pupae supplement and dried silkworm pupae powder. Depending on the number of silkworm pupae added to the medium, the growth rate of the mycelium system is different. The time of starting to appear fruit bodies of the formulas was also significantly different; formula six and formula 7 had the fastest time to appear fruit bodies (12 days), and the slowest was formula 1 (19 days). The size of the fruit bodies is also highly dependent on the number of nutrients present in the culture medium. The more nutritious the medium is, the larger the fruit body size is. If the medium has too many nutrients, the height of the fruit bodies will be underdeveloped, and only the diameter of the fruit bodies developed (as in formula7). From the above analysis, it is necessary to choose an environment that provides just enough nutrients so that the fungi can grow in size in the most balanced way and achieve the best yield and quality. At the same time, it can reduce the cost of raw materials. From the studied media, it was found that formula 7 was the most suitable for the growth and development of the mycelium and the formation of the fruit bodies of *C. militaris.*

CONCLUSION

We have completed the culturing process of *Cordyceps militaris*. The results are summarized as follows:

- 07 culture formulas were established. In which recipe 01 obtained 40 ± five fruit bodies, while formula 07 obtained 60 ± four fruit bodies.
- The number of Escherichia coli bacteria was determined: Our procedure of culture is ensured to be clean, and the presence of Escherichia coli has not been found in the product.

The above are the initial results that show that the research can be put into practice. We suggest that further research is needed to determine the content and develop a complete procedure of culture to obtain artificial Cordyceps with a quality closer to natural Cordyceps.

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