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RESEARCH ARTICLE

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Effect of Abiotic Factors on Fumosorinone Production from *Cordyceps fumosorosea* via Solid-State Fermentation

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ABSTRACT

Cordyceps fumosorosea is an important species in the genus of *Cordyceps*, containing a variety of bioactive compounds, including fumosorinone (FU). This study was a ground-breaking assessment of FU levels in liquid and solid cultures. The present study focused on the impacts of solid-state fermentation (SSF) using solid substrates (wheat, oat, and rice), as well as the effects of fermentation parameters (pH, temperature, and incubation period), on the generation of FU. All the fermentation parameters had significant effects on the synthesis of FU. In a study of 25 °C, 5.5 pH, and 21 days of incubation period combinations calculated -to give maximal FU production, it was found that the optimal values were 25 °C, 5.5 pH, and 21 days, respectively. In a solid substrate medium culture, FU could be produced from SSF. At 30 days, a medium composed of rice yielded the most FU (798.50 mg/L), followed by a medium composed of wheat and oats (640.50 and 450.50 mg/L), respectively. An efficient method for increasing FU production on a large scale could be found in this approach. The results of this study might have multiple applications in different industrial fermentation processes.

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Cordyceps fumosorosea; fumosorinone; abiotic factors; solid-state fermentation

1. Introduction

The use of *cordyceps* as a traditional medicine and healthy food in Asian countries has been around for a long time [1,2]. As well as being widely used in agriculture for biological pest control, some *Cordyceps* fungi are also widely used in medicine [3,4]. Because of its long-term co-evolution with insects, the *Cordyceps* fungus may be capable of producing a wide range of bio-active compounds, including cordycepin, militarinone, myriocin, cyclosporin, destructxins, fumosorinone, enniatins, among others [5].

Since the beginning of time, insect pathogenic fungi have been considered potential agents for the biological control of a variety of insects. Kepler et al. regrouped *Isaria fumosorosea*, *Spicaria fumosorosea* and *Paecilomyces fumoroseus* into *Cordyceps fumosorosea* [6–8], showed wide geographical distribution and strong ecological adaptability. *C. fumosorosea* is easy to culture, has a fast growth rate, can produce spores in a short time, and is widely used in biological control. a highly effective and economical insecticide due to its wide range of insecticidal activity, low cost of production, and safety for humans and other non-target species related to its wide host range [9]. There are, however, complications with *C. fumosorosea*, as it takes some time to take effect after practical application and is easily affected by its surroundings. Recent findings indicate that nanoparticles of *C. fumosorosea* can cause effective control of different insect pests [10,11].

As one of the most widely known entomopathogenic fungi in the world, *C. fumosorosea* is capable of killing many pests and synthesizing a variety of biologically active compounds, including beauvericins, beauverolides, cepharospirolides, trichocarnanes, FU among others [12,13]. FU is signature metabolite of the species, and there were many publications on FU, a novel inhibitor of PTP1B, that activates insulin signaling in insulin-resistant HepG2 cells and shows anti-diabetic effects in diabetic mice [14]. It was found in our previous study that FU inhibited the activity of protein phosphatase 1B, the enzyme that is necessary to secrete body fat. FU was isolated from *C. fumosorosea*, which is an insect pathogenic fungus FU inhibited PTP1B activity [15]. Physiologically, PTP1B is an antagonist that inhibits the insulin signaling pathway in this way, which

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may result in increased sensitivity of insulin [16]. PTP1B has been regarded as a potential therapeutic target for preventing and treating insulin-induced illnesses [17]. The FU, a novel chemical derived from insect pathogenic fungus, has been demonstrated to reduce the proliferation, mobility, and penetration of breast cancer cells MDA-MB-231 when used in conjunction with this compound [18] Liu et al. identified the synthetic gene clusters of FU, and found that the biosynthetic gene clusters were PKS-NRPS gene, two cytochromes P450 enzyme genes, one trans-alanyl reductase gene and two transcriptional regulatory genes, and made reasonable speculations on the synthesis process. However, there are few reports on the transcriptome and metabolomics in *C. fumosorosea*, so the expression and regulation of the fumosorinone FU gene are less clarified, which is one of the reasons for the few studies on FU enrichment. Culture conditions have a significant influence on the Cordyceps metabolic process including temperature, pH, incubation time and medium type. Many researchers are committed to improving cultural conditions to obtain better results. For example, deep liquid fermentation technology can effectively enrich cordycepin in *Cordyceps militarists* [19]. Cordycepin's optimal culture temperature is 25 °C, and the yield at 30 °C is only about 1.5% of the optimal temperature [20]. PH and temperature affect the enzymatic reaction. The metabolic process of organisms has its time concept, and the content of metabolites in different growth stages is different, so it is crucial to choose the right time of cultivation. Other researchers have found that suitable grains such as rice, wheat and oats can promote biomass and active matter accumulation to varying degrees [21,22]. Selecting a suitable medium according to the biological function and effluence of metabolites is helpful to better obtain the desired product.

To sum up, *C. fumosorosea* is a species with great potential for development, and FU is a drug to treat diabetes. The previous experimental observation showed that the biomass obtained by solid fermentation was significantly higher than that obtained by liquid culture, so solid fermentation was used as the final research substrate. The purpose of this study was to screen the optimum culture temperature, pH and time through multiple single-factor experiments, and then compare the yield of the three solid substrates on this basis, to obtain the maximum FU yield.

2. Materials and methods

2.1. Culture preparation

Cordyceps fumosorosea was collected from Dayao County, Chu Xiong City, Yunnan Province. A certain amount of sporangium powder was selected and inoculated into a PDA medium (potato 200 g/L, dextrose 20 g/L, agar 20 g/L), and the strains were trans-purified several times to obtain pure culture [23]. DNA of pure culture was extracted by the CTAB method, and ITS and tef-1αgene fragments were used to identify the species. *C. fumosorosea* cultures were produced and inoculated to optimize the parameters of solid-state fermentation (SSF), it was necessary to select pH, temperature, incubation time, and other parameters, basal medium is used in these processes, the formula was composed of (glucose ($C_6H_{12}O_6$) 1.5%, peptone ($C_{13}H_{24}O_4$) 0.5%), Monopotassium phosphate (KH_2PO_4) 0.3%, Dipotassium phosphate (K_2HPO_4) 0.1%, magnesium sulfate ($MgSO_4$) 0.05%). Using 20 g of rice, wheat, or oats as the solid substrate and 32 mL of the basal medium solution, the composite solid medium for *C. fumosorosea* was created. The *C. fumosorosea* culture was placed in the Yunnan Herbal Her-barium (YHH) of Yunnan University. Yunnan Fungal Culture Collection (YFCC) at Yunnan University maintains a wide collection of fungi cultures, including those from Yunnan province. The strain is preserved in Yunnan Fungal Culture Collection (YFCC), Yunnan University, and related specimens were deposited in Yunnan Herbal Herbarium (YHH), Yunnan University.

2.2. Effect of fermentation environments for FU production

To assess the influence of fermentation conditions, several factors such as pH, temperature, and incubation duration were chosen. As described below, each factor was studied independently.

2.3. Effect of pH

To investigate the ideal pH for FU production, *C. fumosorosea* was determined in 250 mL flasks containing 100 mL of basal medium, with the pH range being varied from 4.0 to 8.0 (4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0). The pH of the medium was changed using a 1 N HCl or 1 N NaOH solution. The flasks were maintained in a static condition at 20 °C in an incubator for 20 days.

2.4. Effect of temperature

During fermentation, the temperature at which FU can be produced optimally was determined by combining 100 mL of basal medium with an initial pH of 5.5 in 250 mL conical flasks with 25 °C intervals, 25 °C, 30 °C, 35 °C, and 40 °C, respectively, and then being kept at 5 °C intervals for 20 days under static

conditions (10 °C, 15 °C, 20 °C, 25 °C, 30 °C, 35 °C, and 40 °C).

2.5. Effect of incubation time

Fermentation was carried out for a period of 30 days under static conditions in a 250 mL flask filled with 100 mL basal medium with an initial pH of 5.5 at 25 °C in order to find out the most efficient incubation time. In order to find out how much FU is produced, 3 days intervals of up to 30 days were monitored for FU production.

2.6. Solid-state fermentation

In order to optimize the parameters of solid-state fermentation (SSF), the three optimal factors screened above were considered. The composite solid medium was used for cultivation, 12–15 days after the temperature was decreased to 16 °C at night while maintaining the culture temperature at 25 °C during the day, the light intensity was retained at 500 lux, and the relative humidity (RH) was maintained at 90–95%, the first form of fruiting bodies started to appear. The chamber's CO_2 levels were kept constant by carrying out sufficient air exchange, and the relative humidity was kept between 80 and 90% while the ambient temperature was kept at 23°. Illumination with an intensity of 300 lx did not exceed 12 h daily, and the relative humidity was maintained at 80–90%. The experiments were conducted with wheat, oats, and rice as the test solid substrates. In order to determine the optimal incubation time, the time sequence analysis of samples cultured by rice was carried out to monitor the growth state and FU yield.

2.7. Extraction and evaluation of FU

After cultivation is finish, the mycelium and the spore bundle were taken out, and then the samples were rinsed 2 ~ 3 times with distilled water and dried at 50 °C in the dryer to constant weight, pulverized with a grinder, sifted through 80 mesh, and finally the powder took the sifted for later use. For the extraction of FU, fermented foods comprising fungal biomass were combined with methanol at a ratio of 1:5. Using a solicitor's assistance, we ruptured the cells by applying a voltage of 100 volts in the range of 100 to 240 volts at 50/60 Hz, at 1.5 Amps, for 10 min, with a pulse of 10 s on and 5 s off. In order to separate each sample, the mixtures were centrifuged for 20 min at 2795 RCF using a Hitachi, HIMAC-CR22N centrifuge, in Japan. Before performing an HPLC analysis on the supernatant obtained from each sample, to removed the supernatant from each sample, filtered it, and then put it in microcentrifuge tubes. An HPLC method was used to analyze FU from different extracts of samples using a C_{18} column (Phenomenex Hyper Clone^{TM} 5 µm BDS C_{18}, United Kingdom) as well as an HPLC system (CECIL 1402, United Kingdom) and Isocratic elution of the samples was performed a flow rate of 1 mL/min with water (0.1%) and methanol at a 2:98 ratio. Moreover, methanol and water (0.1% phosphoric acid) was used as the mobile phase. FU was measured by using the UV detector at 414 nm at a maximum retention time of 100%. For the purpose of calibrating using the FU standard, precise amounts of FU standard were dissolved in distilled water to provide a range of FU standard concentrations.

2.8. Statistical analysis

In each and every one of the experiments, three separate trials were carried out. The results were presented as the mean ± standard error of the mean (SEM) of three separate studies ($n = 3$). Graph-pad Prism 8 is Used to draw bar graphs.

3. Results

3.1. Evaluation of fumosorinone production by Cordyceps fumosorosea

An investigation was carried out to assess the production of FU utilizing the basal medium, as well as the impacts of a number of factors, including the pH, temperature, and incubation time, on the production of the highest amount of FU (Figures 1 to 3).

3.2. Effect of initial pH

There is not the slightest shred of doubt in anyone's mind that one of the most critical environmental elements that have an effect on the development of *Cordyceps fumosorosea* is the pH of the medium. In the course of our investigation, we discovered that the production of FU was significantly impacted by a change in the pH value of the basal medium that was present throughout the fermentation process. The maximum output of FU was measured at a pH level of 5.5 when the maximum FU production was 380 mg/L. Further research led to the discovery that the pH range of 5.0–6.0 was optimal for the production of FU.

3.3. Effect of temperature

According to the findings of the present investigation, low temperatures (10 °C, 15 °C, 20 °C, 25 °C,

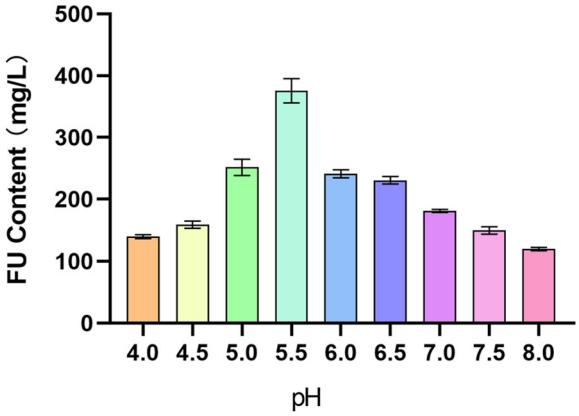

Figure 1. Effect of pH value on FU content. The standard error of the mean is shown by the error bars in the graph.

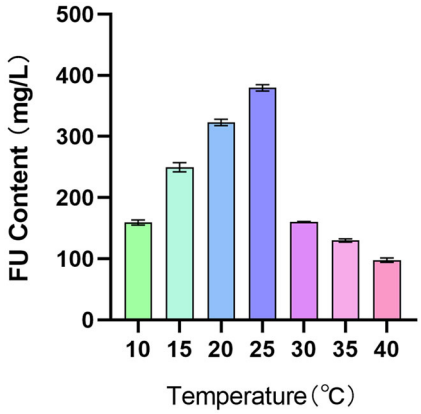

Figure 2. Effect of temperature on FU content. The standard error of the mean is shown by the error bars in the graph.

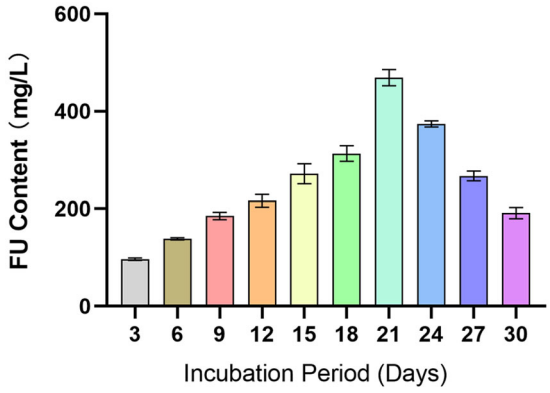

Figure 3. The change of FU content with incubation duration. The standard error of the mean is shown by the error bars in the graph.

30 °C, 35 °C, and 40 °C) were determined to be an unfavorable condition for the production of FU. This was because the production of FU was extremely low, i.e., 167, 250, and 375 mg/L, respectively. In our experiments, we observed that a temperature of 25 °C gave the highest production rate of FU when we used this method. The results of this study support earlier findings that different *Cordyceps* produce FU levels that vary between 50 mg/L and 400 mg/L at a temperature of 25 °C (Figure 2).

3.4. Effect of incubation time

During the course of the experiment, it has been established that FU is created at consistent intervals of 3 days. A significant increase in FU production was observed from 3 to 30 days of fermentation, and it stopped at longer fermentation times. On the 21st day of fermentation in this study, the maximum yield of FU (480 mg/L) was recorded (Figure 3).

3.5. Mycelium growth by solid-state fermentation

Figure 4 shows macroscopic pictures of different stages of rice used as a substrate. In solid culture conditions, *C. fumosorosea* produced sporophore bundles at about 16 days, mycelia thicken at 24 days, and sporophore bundles continue to grow beyond 24 days, At 40d, the sporophore bundles were 5–20cm long, and the colonies were white at first and gradually becomes light pink to pink. The growth and development of the fungus were clearly delayed in comparison to liquid culture conditions. It was found that the highest concentration of biomass and FU was found to be at 30 days (Figures 4 and 5), so the culture time was set at 30 days based on the harvest times for the other two cultures and then the FU content was checked.

3.6. Solid-state fermentation of Cordyceps fumosorosea with grains

Rice, oats, and wheat were the three different basal solid substrates that we evaluated for the generation of FU in the SSF. Our results indicated that rice, oat, and wheat are good substrates forFU production. Within 12 days of the inoculation of the medium, the mycelia had colonized a 300 mL container that contained 20 grams of basal substrate medium, which occurred immediately after the inoculation of the medium. Rice was shown to be the most effective substrate for the production of FU, which enabled a higher overall yield. Rice medium had the greatest amount of FU production (798.58 mg/L) than wheat medium (640.50 mg/L) and oat medium (450.50 mg/L, according to the results of a comparison of FU production among the chosen grains (Figure 6).

4. Discussion

Cordyceps fumosorosea fungi have been used for different biological activities such as controlling pests, controlling breast cancer, and controlling diabetes. FU shows anti-diabetic properties and enormous potential for use in both medical and commercial

Figure 4. Different stages of *Cordyceps fumosorosea* cultured on rice medium. Stage: A: 8d, B: 16d, C: 24d, D: 32d, E: 40d. Scale bars: A–E = 2cm, B = 2cm, C = 2cm, D = 2cm, E = 2cm.

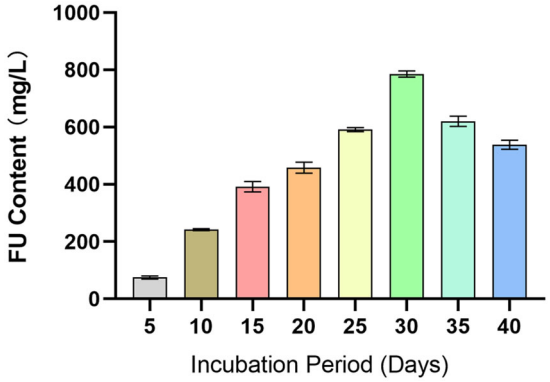

Figure 5. Yield of *Cordyceps fumosorosea* from rice culture at different periods. The standard error of the mean is shown by the error bars in the graph.

applications. One of the key factors contributing to *C. fumosorosea's* rise to prominence as a functional food is its FU concentration. There have been attempts performed to raise the concentration of FU by using pH, Temperature, Incubation time, and

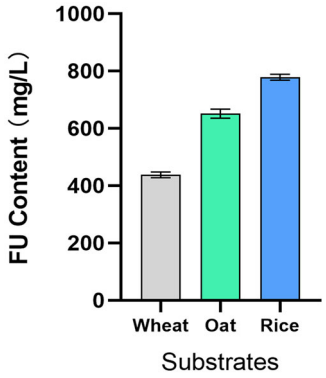

Figure 6. A bar graph showing the impact of several solid substrates on the production of FU. The error bars show the standard deviation from the mean.

Solid-State Fermentation (SSF) in this study. Under liquid culture, the results of a single-factor experiment showed that the optimal pH was 5.5, the optimal culture temperature was 25 °C, the optimal culture time was 21 days, and the maximum yield of FU was 380 mg/L, 380 mg/L, and 480 mg/L, respectively. There is also evidence that variations in pH may affect the metabolism and growth rate of the organisms, as well as the number of nutrients they require for their growth and FU content [21]. The findings of our investigation are in accordance with the findings of earlier studies, which revealed that the pH ranges 4.0–5.5 were optimal for the production of FU at 240 mg/L, the greatest amount that could be produced [24]. FU might not be a growth-associated metabolite. The present study supports previous findings that different *Cordyceps* strains produce FU levels between 50 mg/L and 400 mg/L at 25 °C. The findings of the current investigation were in accord with those reported in earlier studies and published in the literature [25,26]. Interestingly, under the influence of abiotic factors, the metabolite accumulation showed a similar trend as previously reported, for example, the polysaccharide changes in *Ophiocordyceps sinensis* and the cordycepin changes in *C. militaris*, the metabolite accumulation showed a convergence with the growth state [27–29]. There is a big difference in the amount of FU produced using the different substrates. The production of 450.50 mg/L using wheat is significantly lower than the production of 798.58 mg/L using rice. This means that rice is a more effective substrate for the synthesis of FU than wheat. Similarly, the production of 640.50 mg/L using oats is also higher than the production using wheat, but lower than the production using rice. Studies have shown that rice contains significantly more cordycepin and Cordycepic acid. In addition, the C/N ratio of different substrates is significantly different. Wheat's C/N ratio is low, while rice's is high [21], which may be one reason for FU accumulation. In

addition, fungus growth is related to the water content of the substrate, particle gap, and water retention of grain [22], and rice's water retention is higher than that of the other two substrates. Overall, the choice of substrate can have a significant impact on the yield of FU during synthesis, and it is important to carefully consider the substrate used in order to achieve optimal results. It has been demonstrated, with the help of several different *C. fumosorosea* strains, that FU can be produced from spent brewery grains in a concentration range from 100 to 800 mg/L, and this range is dependent on the amount of solid substrate used. This makes the use of cheap grain FU in manufacturing very pertinent. The results of tests employing the most suitable solid substrate in the production of FU from *C. fumosorosea* could be compared to experiments using inexpensive grain [30]. This information is important for researchers and manufacturers who are interested in synthesizing FU for various purposes, such as pharmaceuticals or agricultural applications. By carefully selecting the appropriate substrate. However, this study did not analyze the timing sequence of FU in wheat and oats. The change in FU content in *C. fumosorosea* was unknown. This part will be improved in future research. At the same time, the toxicology and biological function of FU need to be studied to comprehensively evaluate the effects of this compound.

As a result of the findings reported in this study, the FU could be produced on an industrial scale using SSF, and the results obtained could have a significant impact on that. Further exploration is required to pinpoint precisely which physio-chemical element is in charge of the hyper-production of FU in a variety of low-cost food matrixes, which might be useful for future study in this field. Various culture factors (temperature, incubation time, light, shaking time, aeration, and so on) may be studied using different FU strains in order to attain maximal FU content. In addition, the difference in FU production between sporophore bundles and mycelium remains to be studied. As a result of the approach adopted in this study, it is likely that this research will be able to be applied to a wide range of microbial SSF processes. It is believed that further research on the automation of this process is still necessary to improve it.

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Ethical approval

It is declared that there are no competing interests between the authors.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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