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Effect of pH, temperature and incubation time on cordycepin production from *Cordyceps militaris* using solid-state fermentation on various substrates

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ABSTRACT

Cordyceps militaris has been a keystone in combating myriad health problems with innumerable far-reaching therapeutic effects. The present study focuses on effect of fermentation conditions such as (pH, temperature and incubation time) and solid-state fermentation (SSF) using solid substrates (wheat, oat and rice) on production of cordycepin. Temperature, pH and incubation time was found to have a direct effect on cordycepin production. The best possible combination of temperature, pH and incubation time was found to be 25°C, 5.5 and 21 days, respectively, for maximum cordycepin production. SSF of solid substrate medium culture leads to the production of cordycepin. Among the solid substrates, rice medium had highest cordycepin production (814.60 mg/g) followed by oat and wheat medium (638.85 and 565.20 mg/g, respectively). This method provides an effective way for increasing the cordycepin production at a large scale. This study could have a wide application in other fermentation processes at industrial level.

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PALABRAS CLAVE
Hongo; *Cordyceps militaris*; cordicepina; fermentación en estado sólido; proceso de optimización

Efecto de la variación del pH, la temperatura y el tiempo de incubación en la producción de cordicepina a partir de *Cordyceps militaris*, usando la fermentación en estado sólido para varios sustratos

RESUMEN

El uso de *Cordyceps militaris* se ha convertido en piedra angular para el combate de numerosos problemas de salud, identificándose que su empleo produce numerosos efectos terapéuticos de amplio alcance. El presente estudio se centra en los efectos que las condiciones de fermentación de este hongo, entre ellas, el pH, la temperatura y el tiempo de incubación, así como la fermentación en estado sólido (FES) a base de sustratos sólidos (trigo, avena y arroz), tienen sobre la producción de cordicepina. Se constató que tanto la temperatura, como el pH y el tiempo de incubación tienen un efecto directo sobre la producción de cordicepina. Se comprobó que la mejor combinación posible para generar máxima producción de cordicepina es aquella que reúne 25°C de temperatura, pH de 5,5 y 21 días de tiempo de incubación. Por otra parte, se constató que la FES en medios de cultivo a base de sustratos sólidos tuvo como resultado la producción de cordicepina, evidenciándose que el medio de arroz generó la producción de cordicepina más elevada (814,60 mg/g), seguido por los medios de avena y trigo (638,85 y 565,20 mg/g, respectivamente). Por lo que, este método resultó una manera eficaz de elevar la producción de cordicepina a gran escala. El estudio puede tener una amplia aplicación en otros procesos de fermentación a nivel industrial.

1. Introduction

Medicinal mushrooms have been known for more than hundred of years for its bio-metabolites (Tuli, Sharma, & Sandhu, 2014a). Cordyceps, a well-known traditional Chinese medicinal mushroom, belongs to the class Ascomycetes (Cui, 2014). Cordyceps typically inhabits over the surface of pupae in winters, leading to the formation of fruiting body in summers justifying its name as 'winter-worm summer-grass' (Wang, Zhang, Hu, Chen, & Qu, 2008). The Cordyceps species are known for its rich source of phytochemical constituents and their biological activities (Cui, 2014). It has been widely used as traditional food tonic and an analeptic for centuries, and its medicinal properties have attracted much attention (Yang, Jin, Ren, Lu, & Meng, 2014).

One of the most important species of *Cordyceps* genus is *Cordyceps militaris*. It has gained significant importance in therapeutics applications (Ma, Song, & Mei, 2015). *C. militaris* have been known to produce a range of therapeutically active compounds like cordycepin, adenosine, cordymin and exopolysaccharides. Among them cordycepin has been ranked an important and active metabolites, which is a kind of nucleoside analogue having structural similarity with adenosine (Gregori, 2014). Cordycepin lacks 3′ hydroxyl group compared to adenosine structures, which makes it more potent compound. It has been reported in various studies and has capability to interfere with various biochemical and molecular processes including purine biosynthesis and DNA/RNA synthesis (Tuli, Sandhu, Kashyap, & Sharma, 2014b). It also demonstrated to have antitumor, antileukemic, antimetastatic, antibacterial, antiviral, antitrypanosomiasis, antirestenosis, immunomodulatory and anti-inflammatory activities (Tang, Qian, & Zhu, 2015).

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Although, *C. militaris* has been commercially produced using solid-state fermentation (SSF) and submerged fermentation for cordycepin production (Das, Masuda, Hatashita, Sakurai, & Sakakibara, 2010). Extraction of cordycepin from the fermented solution or fruiting bodies can be done by using various conventional methods such as pressurized extraction, Soxhlet extraction and reflux extraction using organic solvents (Wang, Meng-Chun, Chao-Kai, Shu-Wei, & Chang-Wei, 2014). Considerable efforts have been made for higher production of cordycepin by screening the different strains and improving the carbon and nitrogen sources of basal medium (Fan, Wang, & Zhong, 2012; Tang et al., 2015). However, there has been little information available on the development and production of cordycepin using *C. militaris* mushroom from various substrates such as rice, wheat and oat for SSF. The aim of this study was to investigate the effect of various parameters such as pH, temperature and incubation time on the production of cordycepin. Subsequently, evaluated parameters were investigated for improved cordycepin production using various solid substrates.

2. Materials and methods

2.1. Procurement and maintenance of C. militaris ATCC 34164

Microbial strain of *C. militaris* 34164 was procured from American Type Culture Collection (ATCC), Manassas, VA, USA. All the fungal strains were maintained as per the protocols given by ATCC.

2.2. Seed culture preparation

The saline spore suspension 4% (v/v) of *C. militaris* 34164 was prepared and inoculated on potato dextrose agar (PDA) petri dish followed by incubation at 20°C for 7 days. The inoculum was prepared by punching out 5 mm of PDA discs with sterilized cork borer. The discs containing cultures of *C. militaris* were inoculated into 250 mL Erlenmeyer flasks with 100 mL of basal medium (glucose 1.5%, peptone 0.5%, KH_2PO_4 0.3%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, NaCl 0.05%) at 25°C on a rotary shaker incubator, Thermo Scientific-Max Q 4000 (1.35 RCF) for 3 days.

2.3. Effect of fermentation conditions for cordycepin production

The various parameters were selected to evaluate the effect of fermentation condition such as pH, temperature and incubation time. Each factor was analyzed individually as described below.

2.3.1. Effect of initial pH

To determine optimal pH for cordycepin production, *C. militaris* ATCC 34164 were cultivated in 250 mL flasks containing 100 mL basal medium with different pH ranges from 4.0 to 8.0. The pH of the medium was adjusted by using 1N HCl or 1N NaOH. The flasks were kept in static mode at 20°C for 20 days in an incubator.

2.3.2. Effect of temperature

In order to evaluate the optimum temperature for cordycepin production, fermentation was carried out in 250 mL conical flasks containing 100 mL of basal medium with initial pH 5.5 at 5°C intervals in the range of 10–40°C (10°C, 15°C, 20°C, 25°C, 30°C, 35°C and 40°C) ± 2°C for 20 days under static condition.

2.3.3. Effect of incubation time

In order to evaluate the best possible incubation time, fermentation was carried out in 250 mL flask containing 100 mL basal medium with initial pH 5.5 and temperature 25°C for 30 days under static conditions. Cordycepin production was examined at 3 days interval up to 30 days.

2.4. Solid-state fermentation

SSF was carried out after optimizing selected parameters such as pH, temperature and incubation time. Fruiting medium of *C. militaris* was prepared by mixing 20 g of rice or wheat or oat as solid substrate and 32 mL of basal medium solution (glucose 1.5%, peptone 0.5%, KH_2PO_4 0.3%, K_2HPO_4 0.1%, $MgSO_4$ 0.05%, NaCl 0.05% with 100 mL distilled water) in a 300-mL bottle covered and sealed with plastic and were autoclaved for 30 min at 121°C and inoculated with 5 mL seed culture followed by incubation at 20°C for 12 days under dark treatment for promoting vegetative growth. Primordia of fruiting bodies began to form at 12–15 days after lowering the incubation temperature to 16°C at night (darkness) with culture temperature maintained at 23°C during the day (the white light maintained at 500 lx) and relative humidity (RH) at 90–95%. While the temperature was maintained at 23°C and RH at 80–90%, sufficient air exchanges were used to maintain CO_2 levels. Illumination with 300 lx intensity did not exceed 12 h per day. All experiments were performed in triplicate. The basal solid substrates were tested for fruiting body and cordycepin production in SSF including wheat, oat and rice (Wen, Li, Kang, Kang, & Hyde, 2014).

2.5. Extraction and evaluation of cordycepin

The fermented foods containing fungal biomass of each of the sample was mixed with methanol for cordycepin extraction in the ratio of 1:5. The cells were ruptured with the help of sonicator (input voltage; 100 VAC–240 VAC @ 50/60 Hz 1.5A amplitude; 100%, time period; 10 min, pulse; 10 s on, 5 s off). The mixture of each sample was centrifuged (HITACHI, HIMAC-CR22N, JAPAN) at 2795 RCF for 20 min. The supernatant from each sample was collected and filtered through 0.2 µm membrane and taken in micro centrifuge tube before HPLC analysis. The extracted cordycepin from different samples were analyzed by HPLC (CECIL 1402, United Kingdom) system using C18 column (Phenomenax HyperClone™ 5 µm BDS C18, LC Column 150 × 4.6 mm), and the mobile phase was methanol and water (0.1% phosphoric acid) in the ratio of 2:98 with the flow rate of 1 mL/min by isocratic elution method (Das, Masuda, Hatashita, Sakurai, & Sakakibara, 2008). The retention time of

cordycepin was measured at λ max 260 nm by using UV detector. Accurate quantities of cordycepin standard (Sigma, USA) were dissolved in distilled water to give various concentrations for calibration.

2.6. Statistical analysis

All the experiments were carried out in triplicates. Results were expressed as mean ± SEM of three independent experiments (n = 3).

3. Results and discussion

3.1. Evaluation of cordycepin production by C. militaris 34164

Cordycepin production was evaluated using the basal medium and the effects of various parameters such as pH, temperature and incubation time were studied for the production of maximum cordycepin (Figures 1–3).

3.1.1. Effect of initial pH

The pH of media is one of the very important environmental factors for the growth of *C. militaris*. Our results showed that cordycepin production was significantly affected by an increase in pH value of basal medium during fermentation. It was found that the maximum cordycepin production reached 381 mg/L when the pH was 5.5. Subsequently, it was recorded that pH value in the range of 5.0–6.0 had good yield of cordycepin. Moreover, the variation in pH may lead to differences in metabolic reactions, growth rate and requirement of nutrient consumption for their growth and cordycepin content (Leung & Wu, 2007). Our results were in accordance with the previous reports, which reported that maximum production of cordycepin 203 mg/L was at pH range 4.0–5.5 (Tuli et al., 2014a).

3.1.2. Effect of temperature

The current study showed that the low temperature (10ºC, 15ºC and 20ºC) was not satisfactory condition for cordycepin production, as the production was found to be very low, i.e. 167, 251 and 294 mg/L, respectively. Our results showed that 25ºC was the suitable temperature for maximum production of cordycepin. This could be implied that cordycepin is non-growth-associated metabolite (Hung, Keawsompong, Hanh, Sivichai, & Hywel-Jones, 2009). Other reports suggested that a range of cordycepin 93–544 mg/L were produced at 25ºC for various *Cordyceps* strains. Results of the present study were found to be in accordance with earlier reports (Si-Min, Mei, Wang-Bin, & Song, 2011).

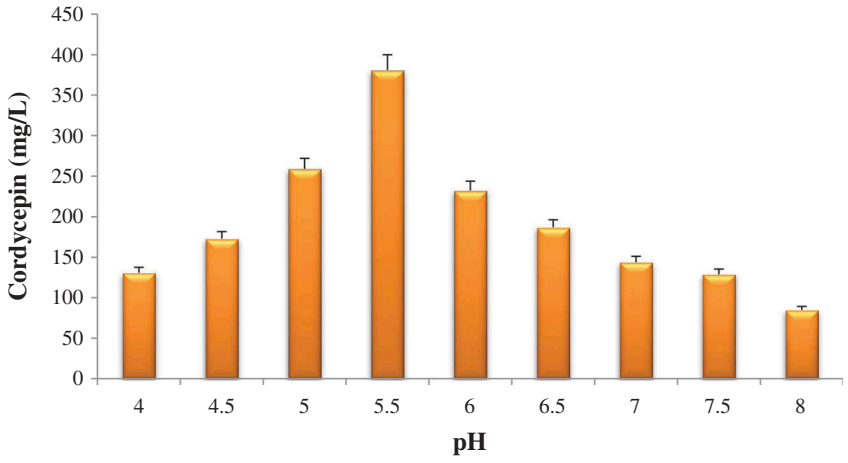


Figure 1. Bar graph showing the effect of pH on cordycepin production. Error bars represents standard error of the mean.

Figura 1. Gráfico de barras que muestra el efecto del pH en la producción de cordicepina. Las barras de error indican el error estándar de la media.

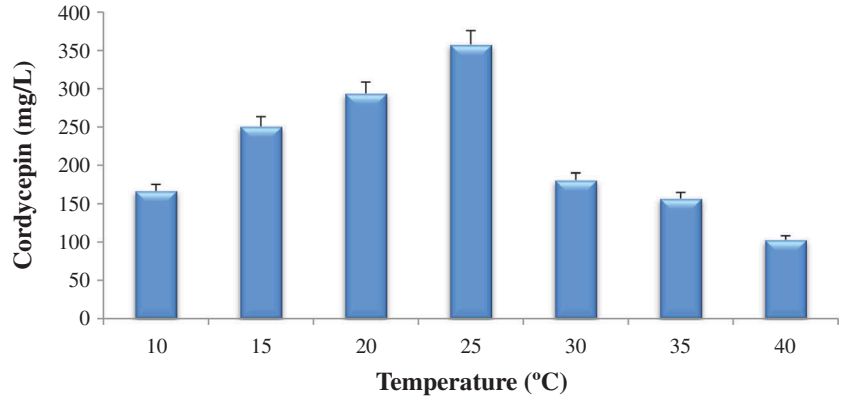


Figure 2. Bar graph showing the effect of temperature on cordycepin production. Error bars represents standard error of the mean.

Figura 2. Gráfico de barras que muestra el efecto de la temperatura en la producción de cordicepina. Las barras de error indican el error estándar de la media.

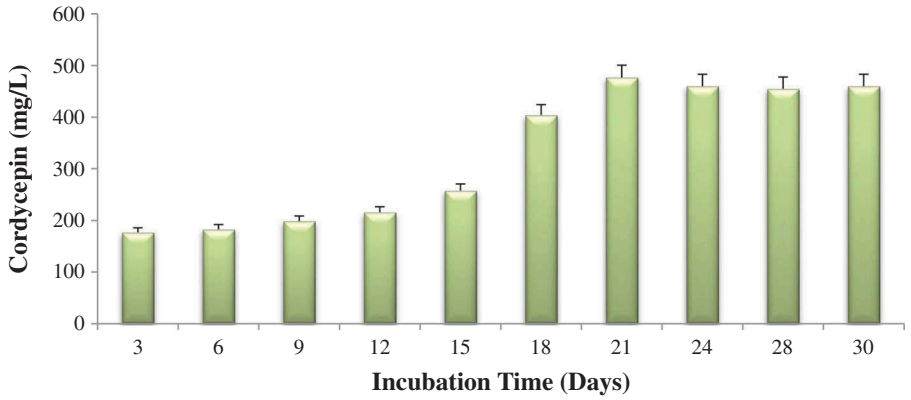


Figure 3. Bar graph showing the effect of incubation time (days) on cordycepin production. Error bars represents standard error of the mean.

Figura 3. Gráfico de barras que muestra el efecto del tiempo de incubación (en días) en la producción de cordicepina. Las barras de error indican el error estándar de la media.

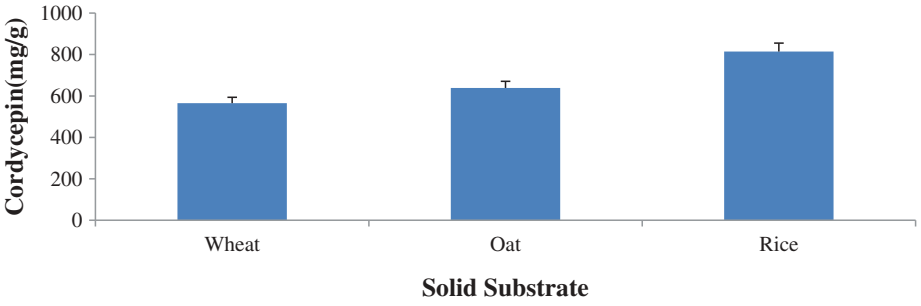


Figure 4. Bar graph showing the effect of different solid substrates on cordycepin production. Error bars represents standard error of the mean.

Figura 4. Gráfico de barras que muestra el efecto de distintos sustratos sólidos en la producción de cordicepina. Las barras de error indican el error estándar de la media.

3.1.3. Effect of incubation time

The cordycepin production was measured at regular intervals of 3 days. The cordycepin production was found to be significantly increased from 3 to 21 days and stopped further with prolonged fermentation time. The highest yield of cordycepin (477 mg/L) was obtained on 21st day of fermentation (Figure 3). Results of the present study were found to be comparable with earlier reports (Si-Min et al., 2011; Tuli et al., 2014a).

3.2. SSF of C. militaris 34164 with grains

In this study, the basal solid substrates for cordycepin production in SSF including rice, oat and wheat were tested and presented in Figure 4. Mycelia entirely colonized 300 mL bottles containing 20 g of basal substrate medium within 12 days following inoculation. The rice was found to be the best basal substrate for cordycepin production. Among the selected grains rice medium had the highest production of cordycepin (814.60 mg/g) compared to wheat (638.85 mg/g) and oat medium (565.20 mg/g). Gregori (2014) reported that the production of cordycepin over spent brewery grains using different strains of *C. militaris* in the range of 100–800 mg/g depends upon the concentration of solid substrates. However, this method was not cost-effective for industrial production. Therefore, the use of low-cost grain to investigate cordycepin production is important. The results obtained in our study were comparable to those using optimum solid substrates to produce cordycepin from *C. militaris* (Wen et al., 2014; Hung et al., 2009).

4. Conclusion

Cordycepin has an enormous potential for medical and commercial use because of its therapeutic activity. However, *C. militaris* has gained importance as a functional food because of cordycepin content. Therefore, efforts have been made in order to increase the concentration of cordycepin using SSF. Wheat, oat and rice are readily available substrates, and the utilization of such substrates leads us to increase in the production of cordycepin 565.20, 638.85 and 814.60 mg/g, respectively. The results obtained in this work could have a significant impact on industrial scale production of cordycepin using SSF. Further research would be fruitful for the exact determination of physiochemical component responsible for the hyper production of cordycepin in different low-cost food matrix. Various cultivation parameters (temperature, incubation time, light, shaking time, aeration, etc.) and their optimization can be investigated for maximum cordycepin content using different strains of *Cordyceps*. The approach used in this study will possibly have a wide application in other microbial SSF processes. However, further research is needed in the direction of automation of the process.

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Conflict of Interest

There is no conflict of interest.

Disclosure statement

No potential conflict of interest was reported by the authors.

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