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Cordycepin production by *Cordyceps militaris* cultivation on spent brewery grains

Proizvodnja kordicepina z gojenjem glive *Cordyceps militaris* na pivovarskih tropinah

Andrej Gregori

Institute for Natural Sciences (Zavod za naravoslovje), Ulica bratov Učakar 108, SI-1000 Ljubljana, Slovenia
Correspondence: andrej.gregori@zanaravo.com

Abstract: This is a first report on *C. militaris* mycelia and fruiting bodies cultivation on solid-state containing spent brewery grains (SBG). Five different strains of *C. militaris* were cultivated on substrates containing rye grains and 0 to 60% SBG. Stromata formation on SBG containing substrates was noticed with two *C. militaris* strains. All strains failed to grow on substrates containing SBG amounts higher than 50%. Highest (10.42 mg/g) cordycepin concentration in cultivating substrate was determined with strain CM2 on 50% SBG. One gram of CM11 strain fungal biomass was able to produce 787.11 mg/g of cordycepin. SBG as a byproduct represent a readily available, low price substrate for cordycepin solid-state production. Obtained concentrations of cordycepin are so far the highest reported concentrations obtained on solid-state substrates therefore we can talk about cordycepin hyperproduction.

Keywords: *Cordyceps militaris*, spent brewery grains, cordycepin, cultivation, medicinal mushrooms

Izvleček: Trosnjake in podgobje glive *Cordyceps militaris* smo gojili na trdih substratih, ki so vsebovali pivovarske tropine. Pet različnih sevov glive *C. militaris* je preraščalo na substratih sestavljenih iz različnih razmerij rži in pivovarskih tropin. Trosnjaki so zrasli pri dveh sevih *C. militaris*. Noben od sevov ni preraščal substrata z vsebnostjo pivovarskih tropin večjo od 50%. Najvišjo koncentracijo kordicepina (10,42 mg/g) v substratu smo določili pri sevu CM2 na substratu s 50% pivovarskih tropin. En gram glivne biomase seva CM11 je proizvedel 787,11 mg/g kordicepina. Pivovarske tropine kot stranski produkt predstavljajo lahko dostopen in poceni substrat primeren za proizvodnjo kordicepina. Dosežene koncentracije kordicepina so po dosedaj znanih podatkih najvišje, zato lahko upravičeno govorimo o hiperprodukciji kordicepina.

Ključne besede: *Cordyceps militaris*, pivovarske tropine, kordicepin, gojenje, zdravilne gobe

Introduction

Cordyceps militaris

C. militaris belonging to *Ascomycota*, is a parasite of insects larval stage, forming fruiting bodies expanding outside the insect larvae or pupae (Buenz et al. 2005). *C. militaris* was traditionally used as a tonic and traditional folk medicine, especially in East Asia (Ying et al. 1987, Holliday and Cleaver 2008, Bhandari et al. 2010). This specie grows wild also in Slovenia (Ogris 2013) and in some other European countries, but its medicinal use in Europe has never been reported.

C. militaris polysaccharides show significant antitumor activities against cervical and liver cancer cells *in vitro* (Yang et al. 2014), extracts of its fruiting bodies show antioxidant, antibacterial, antifungal, antihuman tumor cell lines (Rao et al. 2010, Reis et al. 2013, Yang et al. 2014), anti-inflammatory (Won and Park 2005, Rao et al. 2010), anti-fibrotic (Nan et al. 2001), anti-angiogenetic (Yoo et al. 2004) and insulin secreting (Choi et al. 2004) activities. This fungus is cultivated for cordycepin (3′-deoxyadenosine), a nucleoside analogue with anti-tumour, anti-proliferative, anti-metastatic, insecticidal and anti-bacterial activities (Song et al. 1998).

In recent years *C. militaris* is extensively cultivated in liquid as well as solid media (Das et al. 2010) and is the most successfully cultivated *Cordyceps* species (Kobayashi 1941, Sung 1996). In solid media different supplemented grain types and seeds are used, such as millet, rye, rice, brown rice, bean powder, corn grains, cotton seed hulls, sorghum, corn cobs, jowar, wheat, sunflower floral discs (Chen and Wu 1990, Zhang and Liu 1997, Li 2002, Holliday et al. 2004, Li et al. 2004, Zhao et al. 2006, Gao and Wang 2008, Wei and Huang 2009, Chen et al. 2011, Shrestha et al. 2012, Wen et al. 2014, Yi et al. 2014). SBG so far have not being reported as a substrate component. Wu and coworkers (2013) reported on successful *C. militaris* cultivation and cordycepin production on levan fermentation leftovers. Ni and coworkers (2009) extracted cordycepin from the spent *C. militaris* substrate, concluding it as appropriate source of cordycepin with concentrations ranging from 0.1 to 1 mg/g.

Spent brewery grains

Spent brewery grains (SBG) are a byproduct of the brewing industry which remain as the outer pericarp-seed coat layers from the original malted barley (*Hordeum vulgare*) grain after barley hot water extraction at 65–70°C (Mussatto et al. 2006). SBG are readily available, high volume and low cost byproducts and remain a potentially more valuable resource for industrial exploitation. Currently they are used as an animal feed. Indeed, value-added products are increasingly being sought for SBG (Robertson et al. 2010).

Besides potential uses of SBG for energy via intermediate pyrolysis (Mahmooda et al. 2013), as a potential material for coal production through wet hydrothermal carbonization (Poerschmanna et al. 2014) or a potential candidate for phenolic compounds extraction (Barbosa-Pereira et al. 2014), SBG have been successfully used as a cultivating substrate for *Pleurotus ostreatus* (Gregori et al. 2008), for immobilization of *kefir* and *Lactobacillus casei* for sourdough wheat bread making (Plessas et al. 2007), cultivation of *Lactobacillus plantarum* (Gupta et al. 2013), biomass and xylitol production of *Debaryomyces hansenii* (Carvalheiro et al. 2005). Till now no reports of SBG usage in *C. militaris* cultivation and cordycepin production was reported.

Materials and methods

Cultures cultivation and inoculum preparation

C. militaris cultures CM11, CM14 and CM15 were obtained from Edible Fungi Institute, Shanghai Academy of Agricultural Sciences culture collection, CM2 culture was kindly donated by prof. Wu Wei from Plant Protection Institute, Shanghai Academy of Agricultural Sciences and CM5 culture was obtained from culture collection of Mycomedica d.o.o., Podkoren, Slovenia. All cultures were transferred to Potato Dextrose Agar (Difco, USA) and incubated at 24°C in complete darkness. After mycelium overgrew the agar media, it was homogenized in a blender with 100 ml of sterile water (Wahring, USA). Liquified inoculum was further used for inoculation of cultivation substrates.

Substrates preparation and culturing

Rye (Rebernak, Šmartno na Pohorju, Slovenia) and spent brewery grains (Union d. d., Ljubljana, Slovenia) were mixed in different proportions (9:1, 8:2, 7:3, 6:4, 5:5, 4:6) and filled into 720 ml glass jars. Water was added to the mixture to achieve 65% moisture content and 100 g of substrate weight. Substrates were prepared in triplicates. Jars were covered with metallic lids having 14 mm hole in the middle, covered with HEPA class 14 membrane sterilized for 30 minutes at 121°C and cooled under the flow of sterile air. During inoculation liquid inoculum was constantly mixed on a magnetic stirrer with 5 ml transferred into the substrate jars. Jars were closed and incubated at 24°C under cool white fluorescent light. After incubation the substrate and fruiting bodies were dried for 48 hours at 60°C and milled in a coffee type grinder.

Figure 1: *Cordyceps militaris* (CM2 strain) forming stromata on substrates containing 20% spent brewery grains and 80% rye
Slika 1: *Cordyceps militaris* (sev CM2) tvori podgobje na substratu, ki vsebuje 20% pivovarskih tropin in 80% rži

Cordycepin analysis

10 ml of 20% ethanol was added to 200 mg of powdered sample, and extracted for 2 hours in ultrasonic water bath. The supernatant was centrifuged at 14000 g for 10 minutes and filtered through 0.22 µm membrane filter (Macherey Nagel).

The system consisted of Waters 2695 HPLC system equipped with UV detector. The working

Figure 2. Sterile strain (CM11) of *Cordyceps militaris* not being able to form stromata on substrate containing 20% spent brewery grains and 80% rye
Slika 2: Sterilni sev (CM11) *Cordyceps militaris* ni zmožen tvoriti podgobja na substratu, ki vsebuje 20% pivovarskih tropin in 80% rži

conditions were: YMC - polyamine column (5 µm, 250 mm × 4.6 mm); solvent A - acetonitrile; solvent B - double distilled water; linear gradient - acetonitrile : water (v : v) - (90:10) 15 minutes → (86.5:13.5) 20 minutes → (75:25) 30 minutes → (70:30) 35 minutes; flow rate – 1 ml/minutes; temperature - 30ºC; detective wavelength – 259 nm; injection volume – 10 µl.

Quantification of cordycepin produced by fungal biomass

For determination of ergosterol concentration in control sample 0.2 g of dry *C. militaris* mycelia cultivated on PDA media was extracted in 5 ml of cold absolute ethanol following a modified protocol by Martin and coworkers (1990). For test samples biomass determination one gram of grinded material was extracted in absolute ethanol (10 ml) for 30 minutes at 4ºC, centrifuged at 10000 g for 10 minutes and filtered through a 0.22 µm membrane filter (Macherey Nagel).

Analysis was performed on a Waters HPLC system equipped with PDA 996 detector, 2690 Separation Module and Nucleosil C18, 250 × 4.6 mm, 5 µm column. Ergosterol was eluted at isocratic conditions of 50% methanol and 50% acetonitrile at a flow rate of 1.5 ml/min and

identified with help of standard retention time and the specific absorption triple peak characteristic for ergosterol between 260 nm and 300 nm. For quantification a calibration curve was employed using purified (Nylund et al. 1992) ergosterol standard (Sigma, Germany). Ergosterol content was calculated using calibration curve for fungal mycelia and ergosterol.

Two parameters were calculated for determination of cultivation process effectiveness – cordycepin content in substrate (CCS) and fungal biomass cordycepin production (FBCP). CCS was calculated per substrate weight and shows the end concentration of cordycepin in the substrate (w/w). FBCP shows cordycepin production ability of certain fungal biomass/mycelia quantity (w/w).

Statistical analysis

The data were evaluated by ANOVA (program past 2.16) and significance accepted at $p < 0.05$.

Results

C. militaris mycelia overgrew all the tested substrate mixtures but failed to grow on substrates containing SBG amounts of 60% and higher. When transferred onto SBG containing substrates all strains except CM11 and CM14 formed mycelia with very strong rhyzomorphic primordia forming characteristics. Stromata formation was noticed with strain CM2 (0, 10, 20, 30, 40 and 50% SBG) and CM5 (10, 20, 30, and 50% SBG) (Figure 1).

In all strains except CM11 the increase in CCS is noticed with the increase of portions of SBG in the cultivation substrates. Only in strain CM5 CCS the decrease from 8.90 to 6.64 mg/g was observed in 50% SBG substrate. Maximum CCS (10.42 mg/g) was obtained with strain CM2 cultivated on 50% SBG substrate (Figure 3).

The highest FBCP (787.11 mg/g) was observed with strain CM11 cultivated on 0% SBG drastically reduced (to 305.75 mg/g) with addition of SBG to the substrate (Figure 4). The same FBCP reduction trend at SBG addition was noticed with CM5 strain. FBCP stayed the highest at all SBG concentrations compared to other strains, the second in FBCP was CM5 strain followed by CM2, CM14 and CM15. The lowest FBCP was obtained with CM15 strain on average (Figure 2).

The comparison of different *C. militaris* strains showed that they react differently to SBG addition to the substrate, with CM2 being the strongest

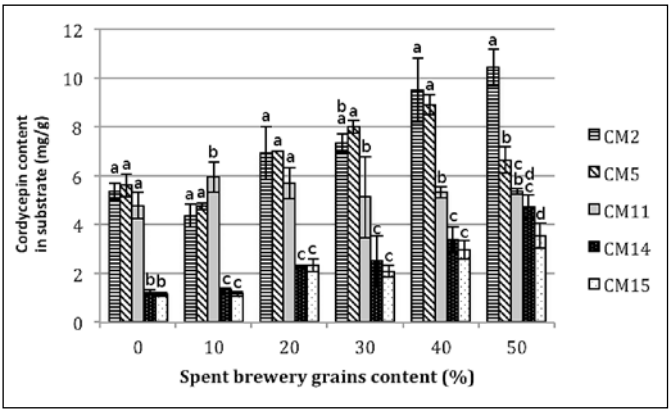

Figure 3: Average cordycepin content in substrates (CCS) containing rye and spent brewery grains overgrown with *Cordyceps militaris*. Columns within a treatment marked with different letters are significantly different

Slika 3: Povprečna vsebnost kordicepina v substratih (CCS), ki vsebuje rž in pivovarske tropine preraščene z glivo *Cordyceps militaris.* Stolpci znotraj enega obravnavanja, ki so označeni z različnimi črkami, se značilno razlikujejo

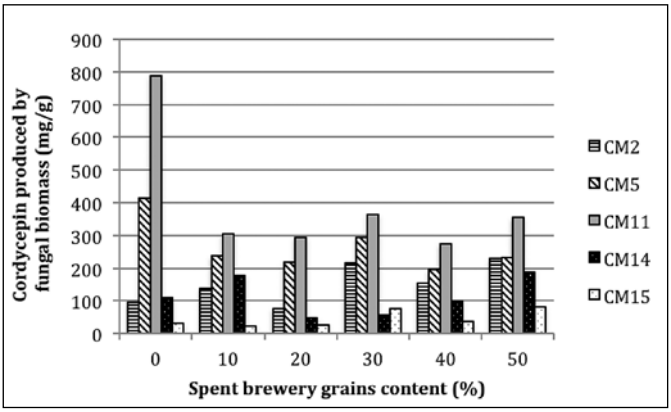

Figure 4. Average *Cordyceps militaris* fungal biomass for cordycepin production (FBCP) on substrates containing spent brewery grains

Slika 4: Povprečna biomasa glive *Cordyceps militaris* za proizvodnjo kordicepina (FBCP) na substratih (CCS), ki vsebuje pivovarske tropine

CCS producer and third strongest FBCP producer. CM11 was the strongest FBCP producer (787.11 mg/g), meaning that 1 g of CM11 biomass can produce up to 787.11 mg of intra and extracellular cordycepin (Figure 4).

Discussion

According to our results SBG addition into *C. militaris* cultivation substrate very effectively increased CCS and at the same time decreased FBCP. CCS hyperproduction in SBG containing substrates could be caused by higher concentrations of low molecular compounds in SBG (simple sugars and other fermentation products produced through the brewing process), compared to unfermented rye grains.

Many different chemically defined substrate supplements are used in commercial *C. militaris* cultivation, with some researchers (Xie et al. 2009) reporting natural substrate components such as brown rice, malt and soybean being better sources of nutrition for *C. militaris* in comparison to chemically defined media. This suggests high cordycepin concentrations in SBG containing substrates could be achieved because SBG is a complex material composed of only natural components.

C. militaris characteristics (white color without stromata forming ability) noticed with CM11 strain were reported by Sreshtha et al. (2012) and is by this author linked to strain degeneration (Figure 2). This could mean that CM11 is a degenerated *C. militaris* strain, capable of producing high FBCP on rye substrate only. At the same time CM11 is the only strain of which CCS is not drastically influenced by addition of SBG to the substrate.

Holliday and coworkers (2004) reported 2.25 mg/g CCS in commercial *C. sinensis* products obtained through solid-state cultivation and 0.65 mg/g cordycepin in wild collected *C. sinensis* stromata. Ni and coworkers (2009) reported 0.1 to 1 mg/g CCS content in spent *C. militaris* cultivating substrates, Wen and coworkers (2014) optimized solid-state composition for *C. militaris* cultivation and achieved CCS of 9.17 mg/g. All reported concentrations are lower compared to results (10.42 mg/g) obtained in our research, showing SBG are a superior, readily available and low cost substrate for cordycepin production through *C. militaris* cultivation.

Why all strains failed to grow on substrates containing SBG amounts of 60% and higher is still unknown. This phenomenon could be linked with higher nitrogen content reported by Gao and coworkers (2000) to suppress *C. militaris* growth.

Conclusion

SBG represent a readily available, low price substrate for cordycepin solid-state production. Here reported concentrations of cordycepin are so far the highest reported concentrations (10.42 mg/g) obtained on solid-state substrates.

Use of SBG for cordycepin production by *C. militaris* is shown here as a very effective technique for producing high value food additive or medicated animal feed - with just drying SBG processed through *C. militaris* cultivation.

Further research are needed to determine the exact components and/or physical properties causing cordycepin hyperproduction in SBG containing substrates and for optimization of cultivation parameters such as temperature, incubation time, light and aeration.

Here described technique of SBG usage is already in the process of optimization and commercialization focusing on high cordycepin content in food and feed production.

Povzetek

Pivovarske tropine predstavljajo lahko dosegljiv, cenen substrat za proizvodnjo kordicepina na trdih substratih. Proizvedene koncentracije kordicepina v substratu so najvišje (10,42 mg/g) koncentracije znane iz objav do sedaj.

Uporaba pivovarskih tropin za produkcijo kordicepina z gojenjem glive *C. militaris* je v članku predstavljena kot enostavna metoda za proizvodnjo hrane, prehranskih dopolnil ali krme z visoko vsebnostjo kordicepina.

Za hiperprodukcijo kordicepina so potrebne nadaljnje raziskave za določitev ključnih karakteristik pivovarksih tropin ter optimizacijo gojitvenih parametrov kot so temperatura, svetloba, trajanje inkubacije in prezračevanje.

Opisane tehnike gojenja *C. militaris* so že v procesu optimizacije in komercializacije za namene proizvodnje prehranskih dopolnil in medicinirane krme z visoko vsebnostjo kordicepina.

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