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OPTIMIZATION OF CULTURE CONDITIONS FOR SINGLE CELL CULTURE OF *Cordyceps militaris* DMR 1164

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ABSTRACT

Cordyceps militaris **produces one of the most important bioactive compound cordycepin. It is quite challenging to synthesize the substantial quantities of this compound for industrial use. The present study was aimed to optimize some cultural conditions of *C. militaris* for maximum mycelial growth, fruiting body formation and cordycepin production. In this study, *C. militaris* DMR 1164 was subjected to serial dilution and 500 µL mycelium was plated onto the potato dextrose agar (PDA) plates and incubated at 22°C for 7 days in dark to isolate single cell colonies. Randomly purified single cell mycelial colony of *C. militaris* was subjected to optimization studies to improve its growth potential. The study revealed the optimal temperature and pH for *C. militaris* mycelial growth to be 20ºC and pH 6. The modified PDA medium No. IV was found ideal medium for achieving luxuriant mycelial growth as compared to other five test media. Liquid medium was suitable for quick mycelial growth than solid medium; whereas, brown rice based solid medium was optimal for fruiting body formation when incubated under light/dark alternate cycles for 65 days. The amount of cordycepin, determined spectrophotometrically at 460 nm, was 8.1 ± 1.32 mg g^{-1} fruiting body. The large scale production of cordycepin can possibly be achieved by exploiting the above studied conditions.**

Keywords: Cordycepin, fruiting body, potato dextrose agar

INTRODUCTION

Cordyceps militaris, an entomopathogenic Ascomycotous fungus, is a member of Sordariomycetes, Hypocreales, and Cordycipitaceae and has great medicinal significance (Wijayawardene *et al.*, 2020). Numerous bioactive substances are produced by *C. militaris* which include cordycepin, adenosine, inosine, carbohydrates, amino acids, organic selenium, ergosterol, sterols, cordycepic acid, sterols, mannitol, and pentostatin (Cui, 2015), multivitamins and superoxide dismutase (Wen *et al*., 2009, Li *et al*., 2010). The nucleoside analogue cordycepin (3-deoxyadenosine) is one of the important physiologically active metabolite produced by the species. This medication promotes immunological regulation, and possesses antihyperlipidemial (de Silva *et al*., 2012), antifungal (Sugar and McCaffrey, 1998), anticancer (Yoshikawa *et al*., 2008), antiviral (Hashimoto and Simizu, 1976), antileukemia (Kodama *et al*., 2000; de Silva *et al*., 2013), anti-fibrotic, pneumo-protective and anti-microbial properties (Tuli *et al*., 2014; Wasser, 2014).

For the treatment of patients with refractory acute lymphoblastic leukaemia (ALL) who express enzyme terminal deoxynucleotidyl transferase (TdT), cordycepin is a phase I/II clinical stage

therapeutic candidate (*http://www.ClinicalTrials.Gov*, confirmed in 2009 by Onco Vista, Inc.). To meet the growing demand, chemical synthesis (Kwon *et al*., 2003) and microbial fermentation utilizing *C. militaris* (Cunningham *et al*., 1950) or *Aspergillus nidulans* (Zhang *et al*., 2006) have been used to manufacture cordycepin. Chemically-produced cordycepin pathway is expensive, and troublesome (Ni *et al*., 2009). Further, it is difficult to refine as compared to the fermentation in biology. Therefore, there is a critical necessity to enhance biological methods (Aman *et al*., 2000).

C. militaris is thought to be the only species of genus *Cordyceps* that can be grown commercially for the industrial synthesis of cordycepin, owing to its ease in growth and ability to produce fruiting bodies on artificial media (Lin *et al*., 2017; Sun *et al*., 2018; Chamyuang *et al*., 2019). Fermentation in solid state (time for fermentation is too long via fermentation in a solid state) [Wen *et al*., 2008; Chen *et al*., 2010], submerged culture (production is typically low, expensive, and susceptible to contamination) [Mao and Zhong, 2004; Mao and Tu, 2005; Wen *et al*., 2010; Zhang *et al*., 2011] and surface liquid culture (surface culture techniques have higher productivity as opposed to other alternative procedures and are less expensive) [Sari *et al*., 2016] in microbial fermentation has been performed to produce cordycepin. The present study was aimed to enhance the *C. militaris* DMR-1164 mycelial formation by employing single cell culture, and optimize some cultural conditions (like growth medium composition, surface area, temperatures and pH) for better mycelium formation in solid substrates so as to improve cordycepin production.

MATERIALS AND METHODS

Cordyceps militaris strain DMR 1164 used in this study was procured from the Directorate of Mushroom Research, ICAR, Solan, HP (India). The 1 mL medium (as rehydration fluid) having composition (L^{-1}) as: peptone 5 g; yeast extract 3 g; and $MgSO_4.7H_2O$ 1 g was used to dissolve the mycelium of this strain. Dilution method was followed to isolate the single cell colonies from cell suspension. A liquid medium comprising of (L^{-1}) yeast extract 45 g; glucose 40 g; KH_2PO_4 0.5 g; K_2HPO_4 0.5 g; $MgSO_4.7H_2O$ 0.5 g, and $FeSO_4 \cdot H_2O$ 0.1 g was used to serially dilute the mycelia. Diluted mycelium (500 µL) was plated on potato dextrose agar (PDA) and incubated at 22 °C for 7 days in dark. After incubation and mycelial growth, a single cell colony was isolated and placed onto fresh PDA medium in Petri-plates and slants. The plates were used for cultivation in liquid medium, whereas, PDA slants were stored at 4°C to maintain stock culture.

Cultivation of C. militaris single cell colony on different media

C. militaris DMR 1164 single cell colony was transferred to six different culture media by punching out 5 mm agar plate culture with the help of a sterilized agar cork borer to assess its optimum mycelial growth medium for about 12 days in dark at 20-22ºC. The composition of culture media used to assess optimum mycelial growth by *C. militaris* DMR 1164 was as under:

Media	Dextrose (g)	Peptone (g)	Yeast extract (g)	Citrate (g)	$MgSO_4$ (g)	KH_2PO_4 (g)	Potato extract (mL)	Sucrose (g)	Water (mL)	NH_4SO_4 (g)	KNO_3 (g)	$CaCl_2$
I	30	5	3	0.1	0.04	0.1	-	-	-	-	-	-
II	30	3	-	1	1	2	-	-	-	-	-	-
III	-	-	-	-	-	-	200	40	800	-	-	-
IV	20	-	4.4	-	2.4	5	200	-	-	1.2	1.2	Trace
V	-	20	-	-	0.1	0.01	-	20	-	-	-	-
VI	40	-	-	-	-	-	200	-	800	-	-	-

Effect of surface area, temperature and pH on mycelial growth

Single cell colony of *C. militaris* DMR 1164 was grown in sterilized wide plastic boxes (of 22.5 mm x 12.3 mm x 7.7 mm of 2 L volume with increased surface area). The growth was compared with 300 mL volume glass bottles (with less surface area), to observe the effect of surface area on

mycelial growth of *C. militaris* on modified PDA medium No. IV (brown rice based solid medium) at 20ºC, pH 6. *C. militaris* was grown at varying temperature from 10 to 35ºC (i.e. 10, 15, 20, 25, 30 and 35ºC) and pH varying from 3 to 9 (*viz*., 3, 4, 5, 6, 7, 8 and 9) to determine the optimal mycelial growth conditions after 4-8 days in dark condition. The experiment was also done under alternate dark and light conditions too. All the three experiments were performed three times.

Seed culture and fruiting body formation

Mycelial growth was tested in liquid surface culture by comparing it with solid substrate culture method. *C. militaris* DMR 1164 seed culture was prepared by taking 5 mm culture grown on modified PDA medium No. IV in Petri-plate and transferring it into a 250 mL conical flask containing 50 mL sterilized liquid modified PDA medium No. IV and incubating in a rotary shaker incubator at 20ºC, pH 6 at 150 rpm for 10 days (without rotation for 15 days). After incubation, 7 mL seed culture (liquid spawn) was inoculated on sterilized modified PDA medium No. IV (40 mL) with either of one solid substrate [brown rice /soya seeds (20 g)] and incubated for 15 days in dark at 20ºC with relative humidity (RH) of 60-70% for mycelial growth. The fruiting body formation was stimulated by following alternate dark/light cycles (12 h each) for 45 days with RH of 80-90% at 20ºC.

Extraction and identification of cordycepin

Fruiting body was used to extract cordycepin with initial water extraction at 85°C for 2.5 h and then ultrasonic extraction at 600 W for 35 min (Zhang *et al*., 2011). The extracted crude material was diluted with n-butanol in 1:2 ratio. The cordycepin fraction was identified by spotting extract on thin layer chromatographic (TLC) plates. The TLC run was performed with solvent system comprising of chloroform/ methanol/ water (64:14:1), The compounds were visualized in UV chamber at 260 nm or by spraying 10% H_2SO_4 in ethanol followed by heating on hot plates. The retention factor of cordycepin (*R*f) was 0.46 (Sari *et al*., 2016). The compound was scratched out from TLC plate and quantified spectrophotometrically at 460 nm (Krishna *et al*, 2024).

All the experiments were performed in a completely randomized design and repeated three times. The data generated was statistically analysed (Gomez and Gomez, 1984) and presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Single cell colony growth of C. militaris DMR 1164

After incubation for 7 days in dark, around 150-200 single cell colonies were formed from 500 µL of diluted mycelial suspension of *C. militaris* DMR 1164 on PDA plate (Fig. 1A). Single cell colony of *C. militaris* was grown on PDA plate to allow mycelial growth in dark for 12 days at 20-22ºC (Fig. 1B-C). Culture was grown and saved on fresh PDA plates and slants for future use.

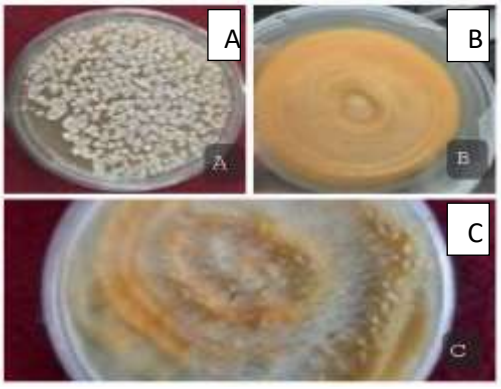

Fig. 1: A) Single cell colonies of *C. militaris* DMR 1164 on PDA plates; Mycelial growth from single cell colony of *C. militaris* in dark on PDA plate; B) after 6 days (Petri- plate back view); and C) after 12 days (Petri-plate front view)

Effect of media composition on mycelial growth

Nutrients are the main factor that greatly impact mushroom development (Nguyen *et al*., 2021). The single cell culture of *C. militaris* was grown on six different media, with fixed pH 6, for 12 days in dark at 20 °C to assess best medium for optimal growth. Among the six different media evaluated, modified PDA medium No. IV showed optimum mycelial growth

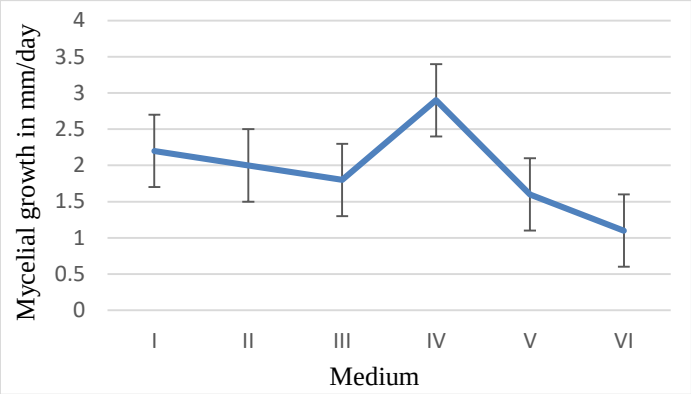

Fig. 2: Mycelial growth of *C. militaris* DMR 1164 on various media in dark

(Fig. 2). The *C. militaris* grown on various media yielded 1 to 14 g cordycepin (Cho *et al*., 2009). Yang *et al*. (2014) by using response surface method found that a submerged culture of *C. militaris* had best mycelial development on medium comprising of yeast extract 10.33 g L^{-1}, sucrose 27.24 g L^{-1}, and KH_2PO_4 5.60 g L^{-1}.

Effect of surface area, temperature and pH on mycelial growth

Single cell colony spawn culture of *C. militaris* DMR 1164 was grown on the modified PDA medium no. IV (brown rice based solid media) optimally (4-5 days) with increased surface area in wide plastic boxes (Fig. 3) than in glass bottles with less surface area (7-8 days) in dark condition. The same result was observed with dark and light alternate cycles too. The *Cordyceps* strain DMR 1164 mycelium grown optimally at 20ºC and pH 6 (Fig. 4, 5) than other temperatures and pH conditions tested.

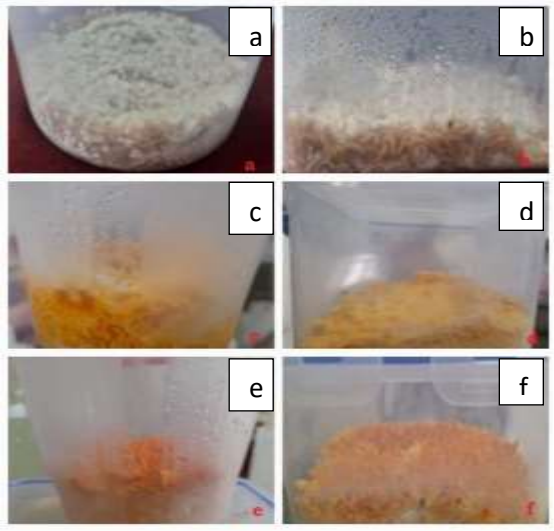

Fig. 3: Effect of surface area on *Cordyceps* growth on modified PDA medium no. IV with brown rice as solid substrate a) after 4 days of dark cycle in wide round plastic box (straight view); b) after 4 days of dark cycle in rectangle shaped plastic box (straight view); c) after 20 days of dark and light alternate cycles in wide round plastic box (straight view); d) after 20 days of dark and light alternate cycles in rectangle shaped plastic box (side view); e) after 40 days of dark and light alternate cycles in wide round plastic box (straight view); f) after 40 days of dark and light alternate cycles in rectangle shaped plastic box (side view)

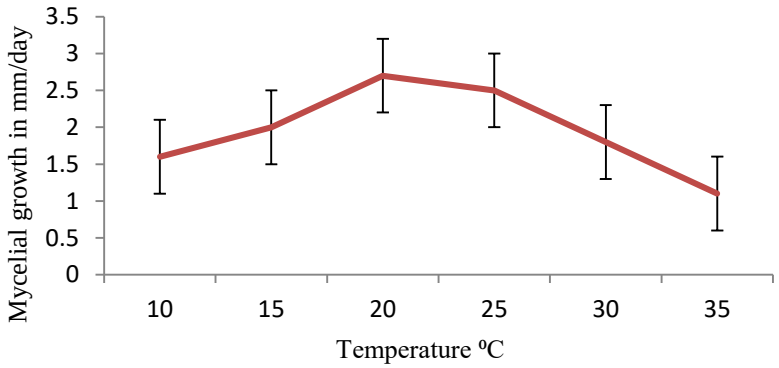

Fig. 4: The mycelial growth (in mm) of *C. militaris* DMR 1164 on modified PDA medium no. IV at different temperatures

Mycelium growth, fruiting body formation and cordycepin production

The mycelium of *C. militaris* DMR 1164 strain grew quickly in liquid medium than in solid medium.

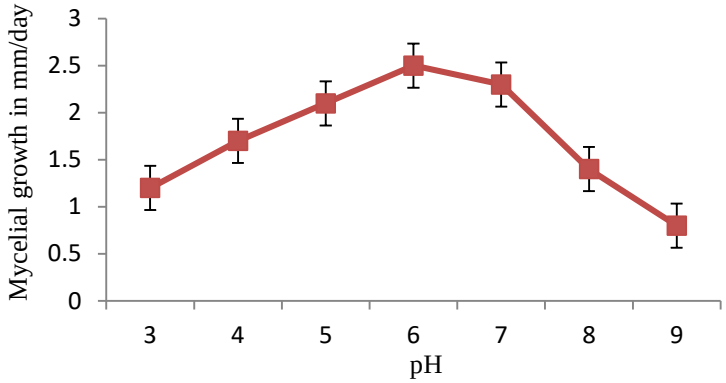

Fig. 5: Mycelial growth (in mm) of *C. militaris* DMR 1164 on modified PDA medium no. IV at different pH values

Initially, the soya seed based solid medium showed more mycelial growth than brown rice based solid medium (Fig. 6-8), but, fruiting body growth initiation was not seen on soya based medium after the light and dark alternate cycles of 20 days. Hence, brown rice based solid culture medium was used for fruiting body formation till 65 days of light and dark alternate cycles.

Fig. 6: *Cordyceps* DMR 1164 strain on modified PDA medium no. IV in dark cycle (4th, 8th and 12th day growth serially): A, B, C: brown rice as solid substrate; D, E, F: soya seeds as solid substrate

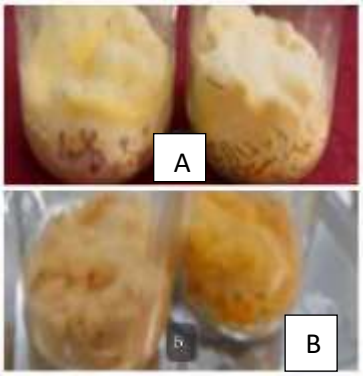

Fig. 7: growth of *Cordyceps* in modified PDA medium No. IV in soya and brown rice as solid substrate; A) after dark/light alternate of cycles 12 days; B) after dark/ light alternate cycles of 20 days

After pinning process the fruiting bodies were formed (Fig. 8), and cordycepin was extracted from fruiting bodies and detected by TLC. The reproducibility of cordycepin production was confirmed with *C. militaris* DMR 1164 strain by employing the test in triplicate.

Fig. 8: *Cordyceps* DMR 1164 pinning process on brown rice solid substrate based modified PDA medium no. IV (A) after 15 days of light and dark alternate cycles (far view) (B) after 18 days of light and dark alternate cycles (close view) (C) after 25 days of light and dark alternate cycles (close view) 8II: *Cordyceps* fruiting body (D) after 33 days of light and dark alternate cycles (normal view) (E) after 45 days of light and dark alternate cycles (close view) (F) after 65 days of light and dark alternate cycles.

Cordycepin extraction and identification

Fruiting body formed by *C. militaris* DMR 1164 (grown on brown rice based solid substrate) was used to extract Cordycepin. It was later on identified by using TLC (Fig. 9) with Rf value of solute peak, comparing with the Rf value of standard cordycepin. The amount of cordycepin determined spectrophotometrically at 460 nm was 8.1 ± 1.32 mg g^{-1} fruiting body. The cordycepin content was found in *C. militaris* strains grown in silkworms was 4.17 ± 1.66 mg g^{-1}, followed by brown rice medium (2.98 ± 1.41 mg g^{-1}), whereas *C. militaris* grown in PDB had the lowest concentration of cordycepin 1.08 ± 0.73 mg g^{-1} (Kang *et al*., 2014). But, according to Song *et al*. (2023) production titer and yields of cordycepin in an engineered *Yarrowia lipolytica* YlCor-18 strain after 168 h of incubation (grown as fed-batch fermentation) were increased to 4362.54 mg L^{-1} and 213.85 mg g^{-1}, respectively.

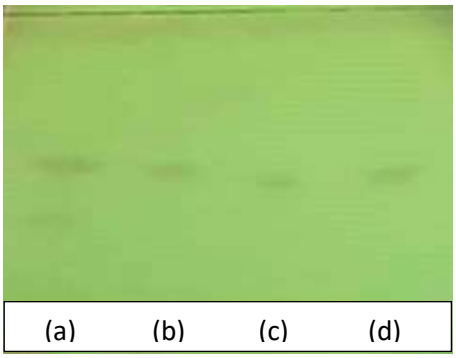

Fig. 9: TLC of cordycepin extracted from fruiting body of *C. militaris* DMR 1164. (a) Extraction with water; b) brown rice extraction with water after fruiting body growth; c) from fruiting body with butanol (d) from brown rice extraction with butanol after fruiting body growth

Cordyceps mycelial growth increased without any mutation steps, using simple single cell colony separation, optimization of media composition, increase in surface area, optimization of temperature and pH. *C. militaris* DMR 1164 mycelium grew quickly on specific culture medium (modified PDA medium no. IV) based on soya as solid substrate. As fruiting body was not seen in soya based medium, brown rice based solid medium was used further for the formation of fruiting body. Temperature at 20ºC and pH 6 for 15 days of dark condition increased mycelial growth and 45 days of alternate dark and light cycles (each 12 h) favoured the fruiting body formation by *C. militaris* DMR 1164. Under above conditions, the production of cordycepin reached 8.1 ± 1.32 mg g^{-1} fruiting body. These findings may have application in larger scale fermentation to enhance cordycepin yield**.**

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Conflict of interest: The authors declare that they have no conflict of interest regarding the publication of this paper.

Authors contributions: SVR, MT prepared and edited the manuscript, whereas AC, BN and ESK carried out the research work. There are no funds granted to pursue this research project.

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