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Effect of Different Parameters on Growth of Strains of *Cordyceps militeris*

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ABSTRACT: *Cordyceps militaris* is a type of fungus that has a wide range of biological benefits. Unfortunately, the wild species of *Cordyceps militaris* has become extinct due to overuse. Therefore, researchers conducted a study to determine the best *in vitro* culture conditions for growing fruit bodies of *Cordyceps militaris*. The fruiting body contains cordycepin, a compound that has several health benefits for humans. Currently, degeneration is the biggest problem of commercial scale cultivation of *Cordyceps militaris*. Screening the best bacteria with high productivity and medicinal value is considered a realistic way to solve the degradation problem. The study investigated the growth of *Cordyceps militaris* mycelium at different temperatures, pH levels and substrates. Seven strains of *Cordyceps militaris* were collected from Sikar, Rajasthan, out of which three strains were selected for further studies based on their sporulation. The mycelium growth of different strains was studied at different temperatures ranging from 20°C to 40°C. It was found that the mycelium growth of all three strains reached its maximum at 25°C, while the minimum growth was observed at 35°C and 40°C. The researchers also adjusted the liquid media at different pH levels ranging from 5.0 to 8.0 and found that the growth was maximum at an alkaline pH of 5.5 and 6.5 for all three strains, while the minimum growth was observed at a pH of 7.0 and 8.0. Finally, the growth of *Cordyceps militaris* mycelium in liquid culture media was studied with different substrates, including brown rice, corn, oats, and wheat. The study found that all three strains of *Cordyceps militaris* grew fastest on brown rice and slowest on wheat and oats substrate.

Keywords: *Cordyceps militaris*, mycelial, fruiting body, cordycepin, Sporulation.

INTRODUCTION

Mushrooms have been considered a delicacy and valued for their medicinal properties by early civilizations such as the Greeks, Egyptians, Romans, Chinese, and Mexicans. According to Chang and Miles (1987), there are over 12,000 different mushroom species, with at least 2,000 of them being edible and used for different purposes (Chang *et al.*, 1999). Around 300 species have been cultivated in experiments, with 60 of them being grown commercially. Mushrooms are worldwide popular as a important food ingredients due to their low calorie as well as so many beneficial ingredients such as polysaccharides, phenolics, and triterpenoids (Bhambri *et al.*, 2022). There are many types of mushrooms, which attract great attention as a healthy foods Many of these cultivated species are both edible and medicinal with characteristic of good aroma and texture. *Cordyceps militaris*, which belongs to the Ascomycetes class, is one of the most important medicinal mushrooms and is widely used as a crude medicine and folk tonic in East Asia, as noted by Ying *et al.* (1987). The name "Cordyceps" is derived from the Latin words "cord" and "ceps", meaning "club" and "head". This fungus is often referred to as "soft gold" in China. It contains various ingredients such as cordycepine, polysaccharides, ergosterol, and mannitol. Due to its diverse efficacy, it is now widely used in many drugs (Song *et al*., 1998; Mizuno, 1999; Nag, 2005).

Cordyceps is found in many parts of the world, from subtropical to temperate regions, including North and South America, Europe, and Asia. The main active ingredient found in the fruiting body of Cordyceps is cordycepin, which was first extracted from *Cordyceps militaris* (Cunningham *et al*., 2015). Cordyceps has been used for various purposes such as anti-inflammatory, antifungal, antihyperlipidemic, and antioxidant. Cordycepin, the main active ingredient in *Cordyceps*, is also a Phase I/II clinical stage drug candidate for the treatment of patients with terminal deoxynucleotidyl transferase (TdT)-expressing refractory acute lymphoblastic leukemia (ALL).

The natural Cordyceps fruit body is very small, and the picking price is high. In recent years, *Cordyceps militaris* has been widely cultivated in liquid and media crops (Das *et al*., 2010). Cultivation of Cordyceps military is mycelium using media (Masuda *et al.*, 2017) has provided greater benefits of cordycepin. Amount of cordycepin in fruiting bodies depends on the strain and substrate used (Kontogiannatos *et al.*, 2022). Outer parts of fruiting bodies of *C. militaris* have the highest concentration of nucleosides, polysaccharides, carotenoids and selenium organic compounds.

Therefore, the major aim of this work was to obtain the best strain of *Cordyceps militeris* as well as optimized the suitable culture conditions on which maximum growth of fruiting bodies could be obtained.

MATERIALS AND METHODS

Fungal Strain and Seed Culture Preparation. *Cordyceps militaris* SC1, SC2, SC3 strains were used in this study after screening which were obtained from Jeevan Dhara Mushroom Research and Development Center, Sikar, Rajasthan and Jeevan Mushroom, Jaipur. Pure mycelial cultures were isolated from fresh fruiting bodies and maintained on PGA (potato glucose agar) slants according to the procedure of Lin *et al.* (2017).

The plates were incubated at 20°C for 7 days and then used to inoculate the seed culture. The inoculum culture medium consisted of the following components: yeast extract, 8 g/L; KH_2PO_4, 1 g/L; K_2HPO_4, 2 g/l; glucose, 8 g/L; $MgSO_4$, 1 g/L. *C. militaris* mycelia were transferred to the inoculum culture medium by punching about 1 cm of culture onto a PDA plate with a sterilized cylindrical cutter. The inoculum culture was cultivated in a 250 ml flask containing 150 ml of liquid medium and incubated at 18°C on a rotary shaker (100 rpm) for 7 days.

Solid Substrate Preparation. Rice (R), Wheat (W), Oat stray (O) and soybean (S) were used as solid substrates for the cultivation of *C. militaris* fruiting bodies. The composition of the medium for the preparation of the solid medium was as follows: yeast extract, 8 g/l; KH_2PO_4, 1 g/L; K_2HPO_4, 2 g/l; glucose, 8 g/l; $MgSO_4$,1 g/l. Thirty grams of each solid substrate was poured into the culture bottles separately, and 25 ml of nutrients were introduced into the culture bottles. The culture bottles were covered with plastic film and autoclaved at 121°C for 15 minutes. Each experiment was performed in culture bottles.

Culture Conditions. Sterilized culture bottles containing solid substrates after inoculation of 2.0 ml of seed culture were incubated in the dark and light-treated with exposure to a light intensity of 1000 ± 20 lux during 40 and 50 days of culture at different stages respectively at the end of the cultivation period, *C. militaris* fruiting bodies were separated from the solid medium and marked (Lek *et al*., 2012).

Effect of various factors on the growth of fruiting bodies of *Cordyceps militeris.* Effect of various factors on the growth of *Cordyceps militeris* fruiting bodies Various parameters were selected to evaluate the effect of fermentation conditions such as temperature pH, and substrate. Each factor was analyzed individually and optimize the culture conditions to obtain the best strain that can produce high amount of cordycepin (Chamyuang *et al.*, 2019).

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 an initial pH of 5.5 at 5 intervals 20ºC, 25ºC, 30ºC, 35ºC and 40ºC for 20 days in static condition.

Effect of initial pH. *C. militaris* was cultured 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 using 1N HCl or 1N NaOH. The flasks were kept statically at 20°C for 10 days in an incubator.

Effect of different substrate on growth. The growth of *Cordyceps militeris* mycelium in liquid culture media varying with different substrate namely brown rice, corn, oats stray &wheat grain.

Table 1: Effect of different temperature levels on radial growth of fruiting bodies.

Temperature	Sporulation**						
	SC-1	SC-2	SC-3	SC-4	SC-5	SC-6	SC-7
20ºC	+++	+++	+++	++	++	++	++
25ºC	+++	+++	+++	++	++	++	++
30ºC	++	++	++	+	+	+	+
35ºC	+	+	+	-	-	-	-
40ºC	-	-	-	-	-	-	-

*Mean of three replication; **Categories of sporulation: Excellent (+++), (++) Good, (+) Below average, (-) No growth

RESULT AND DISCUSSION

Culture conditions such temperature, pH significantly affected the growth rate and metabolite production from fungi. Similar findings were also observed by Lee *et al.* (2009). Effect of initial temperature (20–40°C), pH (5–8) on the growth of fruiting bodies were investigated in Table 2 and 3 respectively. The study found that high temperatures of 30ºC, 35ºC, and 40ºC were not favourable for the growth of cordyceps. The colony diameter of cordyceps was observed to be very low for different strains, ranging between 1mm and 76mm. The most favorable temperature for the maximum production of *Cordyceps militaris* was found to be 25ºC. Our results were also found to be in accordance with previous reports Kunhorm *et al.* (2019). Maximum CV was reported in the SC-3 strain, i.e., 4.13, while it was minimum, i.e., 3.54, in the SC-2 strain. Similar findings were observed by Hung *et al.* (2009). At 25ºC temperature, the growth of various *Cordyceps* strains ranged from 132mm to 139mm. Similar results were also reported by Si-Min *et al.* (2011).

Table 2: Effect of different temperature on the growth of fruiting bodies of *Cordyceps militeris* (Colony diameter in mm).

Temperature(ºC)	Mean Growth of *Cordyceps militeris* strain(mm) mycelium		
	SC-1	**SC-2**	**SC-3**
20ºC	129	125	126
25ºC	134	132	139
30ºC	71	75	76
35ºC	46	45	47
40ºC	1.0	1.0	1.0
SEM	1.561	1.417	1.702
CD.	4.736	4.297	5.162
CV	3.87	3.54	4.13

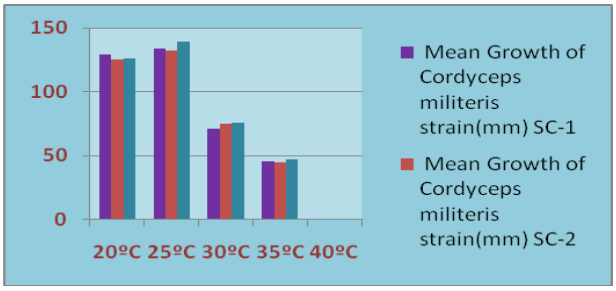

Fig. 1. Showing effect of different temperature on different strains of *Cordyceps militeris.*

The pH level of the media is a crucial factor that affects the growth of *C. militaris*. Studies have shown that the growth of *Cordyceps militeris* in different strains decreases with an increase in pH. The maximum growth of different strains of *Cordyceps militeris* (SC-1, SC-2, and SC-3) was observed at a pH of 5.5, with growth rates ranging between 97 to 101. Previous studies by Tuli *et al.* (2014a) also demonstrated that the maximum growth of cordyceps occurred at pH levels ranging from 4.0 to 5.5. Leung & Wu (2007) additionally reported that pH variation can affect metabolic reactions, growth rate, and nutrient consumption requirements for the growth of cordyceps. Previously Cheng *et al.* (2011); Rózsa *et al.* (2020) also investigated that at a pH of 5.5 favours the highest growth of fruiting bodies of cordyceps.

Table 3: Effect of different pH levels on radial growth (Colony diameter in mm) of fruiting bodies.

PH Range	Mean Growth of *Cordyceps militeris* strain(mm) mycelium		
	SC-1	**SC-2**	**SC-3**
5.0	72	85	84
5.5	101	100	97
6.0	100	96	87
6.5	86	72	75
7.0	73	59	72
7.5	65	74	60
8.0	68	52	51
SEM	2.090	1.563	1.607
CD.	6.339	4.740	4.874
CV	4.88	3.82	4.04

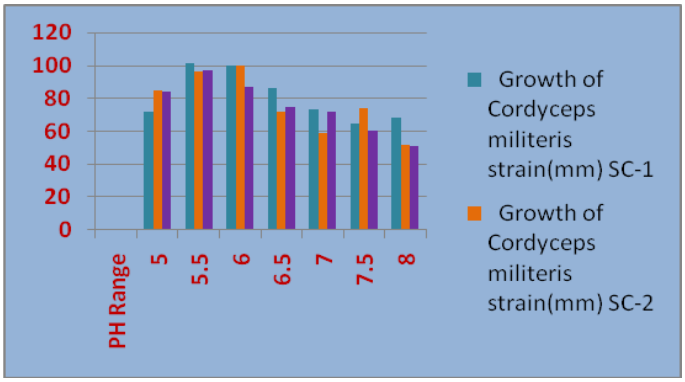

Fig. 2. Showing effect of different pH on the growth of different strains of *Cordyceps militeris.*

Substrate and strain are important factors for the production of fruiting bodies and bioactive components contents in fruiting bodies of *C. militaris*. The present study has observed that the growth of different strains of *Cordyceps militaris* varies depending on the substrate used. Maximum production and quality of fruiting bodies, observed in brown rice substrate i.e. 99mm in SC-1, while oats stray and corn was next suitable substrate, which showed the moderate production of fruiting bodies. However, lowermost production was found in wheat substrate i.e. 59mm in SC-2 strain. Similar results were also observed by (Sharma and Puttoo 2004). Sharma *et al.* (2003) that oat, kutki, and maize grains took the least amount of time for spawn development. Sitara *et al.* (2022) also reported the similar findings. They obtained maximum thick mycelium in brown rice, moderate in oats stray and corn while lowest in wheat. Probable this findings observed due to the higher starch content in rice abd ability to retain water to support the growth and supplement the mycelial growth of *C. militaris.*

Table 4: Effect of different substrate (*brown rice, wheat & oats stray*) on growth of fruiting bodies.

	Growth of *Cordyceps militeris strain(mm)*		
Substrate	**SC-1**	**SC-2**	**SC-3**
Brown rice	99	97	96
Oats stray	80	74	68
Corn	74	61	58
Wheat	61	59	60
SEM	0.925	1.388	1.406
CD.	2.806	4.210	4.265
CV.	2.23	3.59	3.76

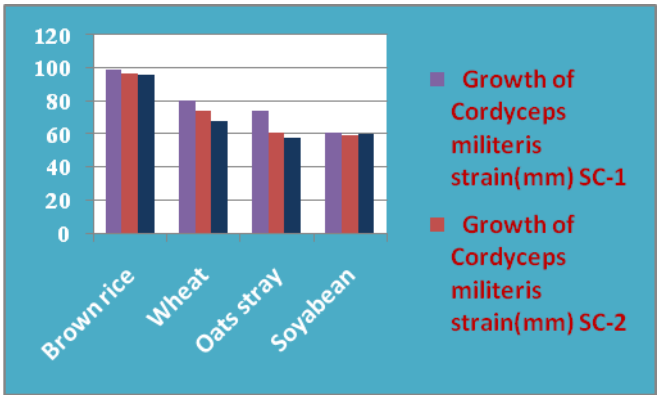

Fig. 3. Showing effect of different substrate on the growth of different strains of *Cordyceps militeris.*

CONCLUSIONS

The growth of *C. militaris* was found to vary with different temperature ranges, pH levels and substrate choices. The temperature range of 20ºC to 25ºC was conducive to mycelium growth, while the optimum temperature for fruiting body production was 25ºC. A pH level of 5.5 and 6.0 was suitable for growth, but the most favourable pH level for growth was 5.5. Brown rice was found to be the most suitable substrate for the growth of Cordyceps. Out of the 7 strains investigated, *C. militaris* SC-1, SC-2 and SC-3 were found to be excellent cordycepin production strains that could be used for commercial production purposes.

FUTURE SCOPE

The strains identified in this study can be commercially exploited to increase cordycepin quality and quantity by producing more fruiting bodies.

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