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CHARACTERIZATION OF AN ENTOMOPHAGOUS MEDICINAL FUNGUS *CORDYCEPS SINENSIS* (BERK.) SACC. OF UTTARAKHAND, INDIA

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ABSTRACT
Higher altitudes of District Pithoragarh, Uttarakhand were surveyed for collection and isolation of the caterpillar fungus, *Cordyceps sinensis*. Fruiting bodies of *C. sinensis* were 4 -7cm long over the caterpillar cadaver ranging 3-4cm in size, mostly erect, stalked, slightly swollen at tip; emerged single, double or triple from the head of larvae. Of the five semi-synthetic media used, SDAY followed by PDA supported maximum growth of the fungus. Hyphae septate branched and 1-30μm wide; colony white to creamish or yellowish with lined depressions, later pink or orange, from reverse purple to purplish brown. Perithecia oval or egg shaped filled with a number of elongated, unitunicate, capitate, cylindrical and hyaline ascus. Optimum temperature and pH for mycelial growth was found to be 15ºC and 6.0.On the basis of these characteristics the fungus was identified as *Cordyceps sinensis* (Berk.) Sacc.

INTRODUCTION

Cordyceps sinensis (Caterpillar fungus) (Wei *et al.,* 2011) a reputed medicinal fungus, (Jing *et al.,* 2011) is an entomophagous in nature. The *Cordyceps sinensis* is basically the costliest medicinal mushroom not only in our country but throughout the world. It has a great pharmacological property (anti-cancerous, anti-asthamatic, anti-HIV property) and is being used for over 2000 years in China for infectious diseases (Jordan *et al.,* 2008).

Ophiocordyceps sinensis (syn. *Cordyceps sinensis*) (Weckerle *et al.,* 2010; Zhong *et al.,* 2010) an ascomycetous fungus, is a parasite on larvae of *Thitarodes (Hepialus)* moths (Winkler, 2008; Shi *et al.,* 2009) (*Hepialus armoricanus*, order-Lepidoptera) and belongs to the family-Clavicipitaceae (Yue *et al.,* 2008) and order Hypocreales (Garbyal *et al.,* 2004; Holliday and Cleaver, 2004; Holliday *et al.,* 2004).

Berkely, the British Mycologist first described this fungus in 1843 as *Sphaeria sinensis* Berk. Later in 1878, Saccardo renamed it as *Cordyceps sinensis*. The accepted scientific name *Cordyceps sinensis* (Berk.) Sacc. is referred to the final form, which is the fruiting body of the fungus arising out of the dead body of a caterpillar (Devkota, 2006).

The *Cordyceps sinensis* ,caterpillar-shaped Chinese medicinal mushroom (Harsahay *et al.,* 2010; Wei *et al.,* 2011)is confined to the high Himalayan mountains in China, Tibet, Nepal and India, at an altitude ranging from 3000 to 5000m (Sharma, 2004) or in Asian high altitude grassland ecosystems (Stensrud *et al.,* 2007).

Cordyceps sinensis consisted of fruiting body and the host caterpillar (Yuan *et al.,* 2007; Jian *et al.,* 2008). The fruiting bodies of caterpillar fungi (dark brown to black in colour) consisted of head parts of various shapes and the 'root' of the organism (the larval body) pervaded by the mushroom's mycelium (Hye Young, 1999; Holliday and Cleaver, 2004).

The culturing of the fungus, *C. sinensis* are feasible on different types of artificial media such as potato dextrose agar, beef extract dextrose agar, casein hydrolysate dextrose agar, soyabean extract dextrose agar, rice extract dextrose agar and finger millet medium (Das *et al.,* 2005; Harsahay *et al.,* 2010). *C. sinensis* fungus cultivated in a liquid medium containing glucose, yeast extract, peptone and few major inorganic salts (Leung and Wu, 2007) and can also be cultured in broth containing carbon sources (rice bran and citrus peel) (Choi *et al.,* 2010). The commercial cultivation can be done in a liquid medium as well as on a solid (grain/potato) phase (Marchbank *et al.,* 2011)

The mycelium of *C. sinensis* was longitudinally, radial and non-aerial and the colour was initially white and later on, densely matted and appeared as orange-brown to tan in colour (Holliday and Cleaver, 2004).The hyphae of *C. sinensis* are

Table 1: Radial growth of *Cordyceps sinensis* on different media

Media	Growth (mm)/Days						Av. growth (mm)/day
	2nd	4th	6th	8th	10th	12th	
SDAY	5.60	14.40	24.40	36.60	41.60	44.93	3.74
PDA	4.20	12.20	18.40	27.40	33.13	40.20	3.35
CDA	2.60	9.20	14.20	20.20	27.80	32.60	2.72
OMA	3.80	11.30	16.70	25.60	32.40	39.13	3.26
MEA	3.00	10.20	15.20	22.80	29.70	36.20	3.01
CD at 5%	Media (a)		-	0.27			
	Days (b)		-	0.29			
	Interaction (a* b)		-	0.65			

Table 2: Radial growth of *Cordyceps sinensis* on SDAY medium at different temperatures

Temp.(°C)	Growth (mm) / Days						Av. growth (mm)/day
	2nd	4th	6th	8th	10th	12th	
5	5.20	13.70	19.50	26.20	31.60	37.67	3.14
10	5.60	14.00	23.20	30.60	36.70	40.10	3.34
15	5.69	14.40	24.40	34.60	41.60	44.87	3.74
20	5.40	13.00	19.00	24.60	30.00	37.50	3.12
25	5.60	12.40	18.70	19.60	24.40	34.27	2.85
CD at 5%	Temperature(a)		-	0.35			
	Days (b)		-	0.38			
	Interaction (a* b)		-	0.85			

Table 3: Effect of pH on radial growth of *Cordyceps sinensis* on SDAY medium at the 15°C temperature

Days	pH levels /Growth (mm)							
	pH_3	pH_4	pH_5	pH_6	pH_7	pH_8	pH_9	pH_{10}
2	5.00	5.40	5.90	6.30	6.00	6.00	5.90	4.00
4	11.30	14.70	14.70	17.00	14.00	15.60	13.50	9.50
6	17.30	24.63	24.80	26.90	24.63	23.63	20.00	14.16
8	21.60	31.60	35.00	37.00	34.20	30.73	25.33	22.20
10	29.60	40.50	41.00	44.93	40.60	36.37	32.50	28.30
Growth/day	2.96	4.05	4.10	4.49	4.06	3.64	3.25	2.83
CD at 5%	Days		0.79					
	pH		1.00					
	Interaction		2.24					

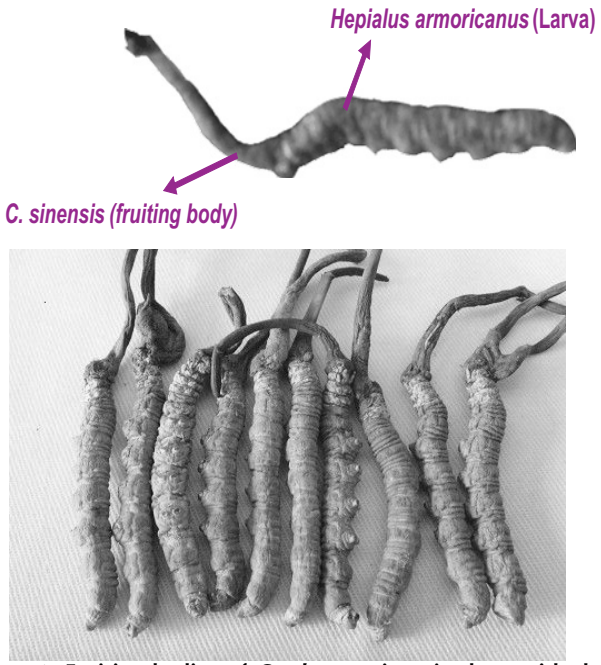


Figure1: Fruiting bodies of *Cordyceps sinensis* along with the mummified body of the insect larvae

white and stout (Xie *et al.,* 2010) and of *C. Khaoyaiensis* and *C. pseudomilitaris* are branched and septate in PDA culture (HywelJones, 1994).The characterization of the *C. sinensis* was done on the basis of the presence of perithecia (Jian *et al.,* 2008) ovoid to cylindrical unitunicate asci (Alexopolous *et al.,* 1996) and ascospores in the ascus (Gwangpo, 2000a; Zhong *et al.,* 2010).

The optimum temperature, 15-20°C and pH, 5 to 5.5 required for its mycelial growth and the fastest growth rate occurred at pH 6.0-6.3 (Gwangpo, 2000b; Das *et al.,* 2005).The initial pH of 6.0 and 7.0 were optimized for submerged cultivation and the culturing of *C. sinensis* in liquid culture, respectively (Yin and Qin, 2009; Jiu *et al.,* 2009).

In our country, the research on the medicinal mushroom (medicinal fungus) is at primitive stage; the medicinal mushroom offers a great hope and also holds a promise for the control of terminal diseases where no complete control is available in the present medicinal systems. There has been not any organized approach for the artificially culturing of *Cordyceps sinensis* in *in-vitro* conditions and its further exploitation for medicinal benefits of the human beings. Due to its peculiar characteristics, habitat, morphology, store house of great medicinal properties, a highly prized mushroom is being harvested from nature every year in a reckless ways,

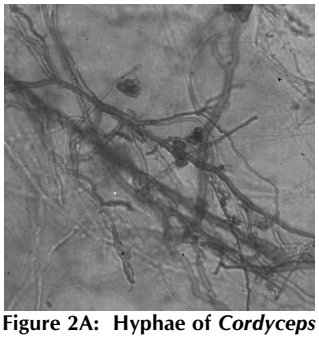


Figure 2A: Hyphae of *Cordyceps sinensis* (60x)

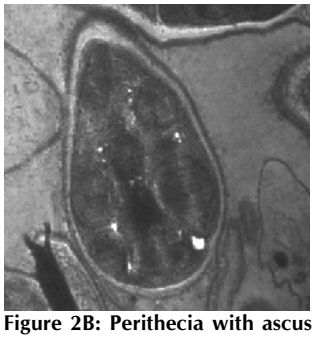


Figure 2B: Perithecia with ascus (resolution 672 x 520 pixels)

leads to extinction in future. The cultivated source will be the better sustainable alternative. Therefore, the present study was undertaken on the basis of survey, by collection and isolation of *Cordyceps sinensis,* characterization on the basis of morphological and cultural studies and the optimization of media, temperature and pH for its artificially cultivation.

MATERIALS AND METHODS

Survey, Collection, Isolation and culture maintenance

The high altitudes of Munsyari and Dharchula, mountain areas of District Pithoragarh, Uttarakhand were surveyed for collection and isolation of the fruiting bodies of *Cordyceps sinensis* during the summer season. The freshly collected fruiting bodies were used for the isolation on the semi-synthetic media *viz*. SDAY (sabouraud's dextrose Agar with yeast extract) and PDA (potato dextrose agar) and the culture maintained as stock culture for further studies.

Morphological and Cultural Characterization

The identification of *C. sinensis* was done on the basis of morphological and cultural characterization. The morphological studies were carried (on the basis of shape, size, colour, etc) of the natural fruiting body and cultural characteristics (perithecia, ascus, pigmentation of the mycelium, media, temperature and pH) required for the growth of the fungus. Transmission electron micrograph of perithecia of *C. sinensis* was taken at different magnification.

Growth on different media

Colony characteristics on five different media *viz*. SDAY (sabouraud's dextrose agar with yeast extract), PDA (potato dextrose agar), CDA (czapek dox agar), MEA (malt extract agar) and OMA (oat meal agar) (Appendix-1) were studied. The stock culture was used as inoculum for the inoculation of the petriplates containing media. For each treatment, three replications were maintained at 15 $\pm$2ºC.

Media, Temperatures and pH for Growth

Five different media enlisted as above were evaluated for radial growth of the fungus at 15ºC.The medium, supported the maximum radial growth was further evaluated at five varying temperatures *viz*. 5ºC, 10ºC, 15ºC, 20ºC and 25ºC.

The effect of pH on the vegetative growth of the fungus was studied on the basal medium at 15ºC.Different initial pH values of medium were adjusted by using pH strips and buffered by using 1.0N HCl or 1.0N NaOH before autoclaving. Seven levels of pH viz. 3, 4, 5, 6, 7, 8 and 9 were maintained. Each treatment was replicated three times and observations were recorded periodically.

RESULTS AND DISCUSSION

Survey, Collection, Isolation and culture maintenance

The different locations present at higher altitudes of Munsyari and Dharchula, mountain areas of District Pithoragarh, Uttarakhand were surveyed to collect the fresh fruiting bodies of *Cordyceps sinensis*. Isolation was done successfully on two media *i.e.* SDAY and PDA from the fresh fruiting body along with larva cadaver, collected from the one location only (village laspa) at the height of more than 3000m elevation. Among the media used for isolation, the best supported medium was found to be the SDAY. Therefore, the medium SDAY was used for maintaining the stock culture for further studies. The feasibility of the growth of the fungus on different artificial media, advocated by Das *et al*. (2005) and Shi *et al*. (2009) are in agreement with present findings as the fungus grew in both the artificial media used for isolation.

Characterization

Morphological Characteristics

Fruiting bodies

The *C. sinensis* consisted of two parts the upper part (ascocarp) - a grass-straw like structure and lower part-the larva cadaver (caterpillar) filled with white mycelium (Fig. 1).

Ascocarp are on an average 4-7cm long over the caterpillar cadaver. The ascocarp are mostly erect, stalked, slightly swollen at tip; emerged single, double or triple from the head of larvae. Stalks are alike grass straw, slightly thickened at the base and tapered towards the end.

The size of caterpillar cadaver varied from 3-4cm. Caterpillar cadaver have worm-like head, body and eight pairs of legs with numerous thin and fine transverse wrinkles.

The similar results were also obtained by the other workers (Yuan *et al.,* 2007; Jian *et al.,* 2008; Harsahay *et al.,* 2010; Wei *et al.,* 2011) reported that the caterpillar shaped chinese medicinal mushroom consists of the fruiting body and the host caterpillar.The fruiting bodies of caterpillar fungi consisted of head parts and parts that look like sacks. The head parts come in various shapes: a circle, a club, a cotton swab stick, a coral reef and noodles (HyeYoung, 1999). The fruiting bodies were dark brown to black and the 'root' of the organism (the larval body) pervaded by the mushroom's mycelium, appears yellowish to brown in color (Holliday and Cleaver, 2004). The root had worm-like head, body and legs with numerous thin and fine transverse wrinkles (Garbyal *et al.,* 2004).

Cultural Characteristics

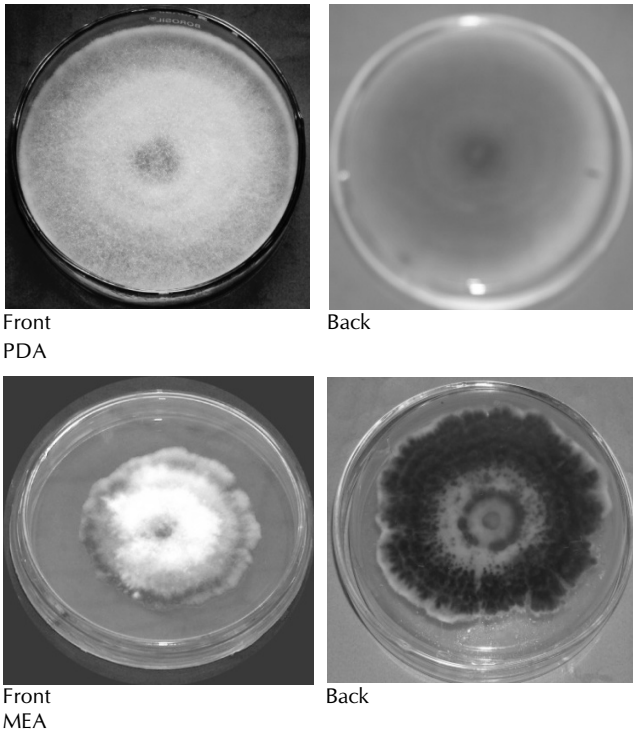
Hyphae

Aerial, cottony white to creamish or yellowish, septate, branched fast growing, finally dense, 1-3μm wide (Fig. 2A).

Colony

The colony characteristics recorded on different media varied to the larger extent and are presented as under (Fig.-3)

Potato dextrose agar (PDA): Colony initially white and later on



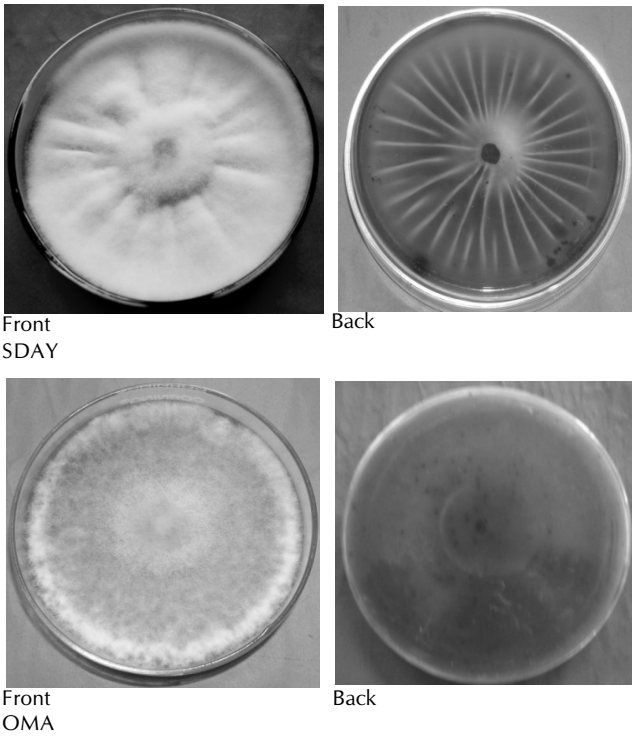


Figure 3: Colony characteristics of *Cordyceps sinensis* on different media

pink red or orange and reverse cream to purplish red in colour.

Sabouraud's dextrose agar with yeast extract (SDAY): Colony initially cream with lined depressions, later dark orange and from reverse dark tan in colour.

Malt extract agar (MEA): Colony initially light pink which was changed to purplish red and from reverse blood red colour.

Oat meal agar (OMA): Colony initially creamish yellow, later light purple and dark tan colour from reverse.

Czapek dextrose agar (CDA): Colony initially light yellow with purplish red margin, finally dark purplish red and dark tan colour in reverse.

The colour as well as the growth and pigmentation of the fungus on media resembled with the description given by the earlier workers (HywelJones, 1994; HyeYoung, 1999; Garbyal *et al.,* 2004; Holliday and Cleaver, 2004; Shi *et al.,* 2009; Xie *et al.,* 2010; Marchbank *et al.,* 2011) referred as above. However, the differences between the shape, size and colour of the fruiting bodies and vegetative growth might be due to location specific.

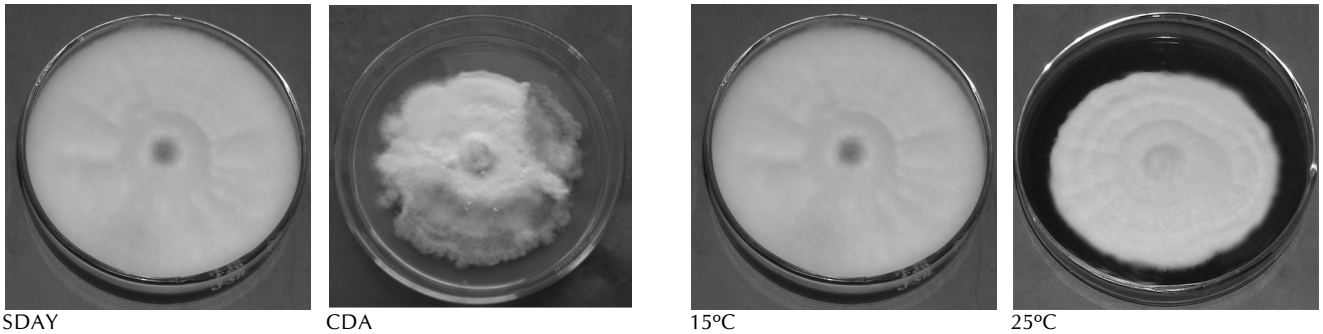
Transmission Electron Microscopy

Transmission Electron Micrograph (Fig. 2B) taken by TEM showed the presence of perithecia and ascus in the samples taken from freshly collected fruiting bodies alongwith insect's cadaver of the fungus *C. sinensis*. Perithecia were found to be oval-shaped or egg-shaped filled with a number of elongated, unitunicate, capitate, cylindrical and hyaline ascus. Perithecia are present in *Cordyceps sinensis* (Berk) Sacc.Our observations are in accordance with the Jian *et al.* (2008) and Alexopolous *et al.* (1996) who showed the presence of perithecia and ovoid to cylindrical unitunicate asci. Gwangpo (2000a) studied the presence of perithecium of *C. sinensis* as either oval-shaped or egg-shaped, consists of countless number of thin, long ascus and ascospores in the ascus. Zhong *et al.* (2010) also reported the ascospores in *ophiocordyceps sinensis*.

The studies conducted employing transmission electron microscope showed the same characteristics of the perithecia earlier reported in case of genus *Cordyceps* of family Clavicipitaceae(Mains, 1958; Rogerson,1970; Gwangpo kim, 2000a and b).

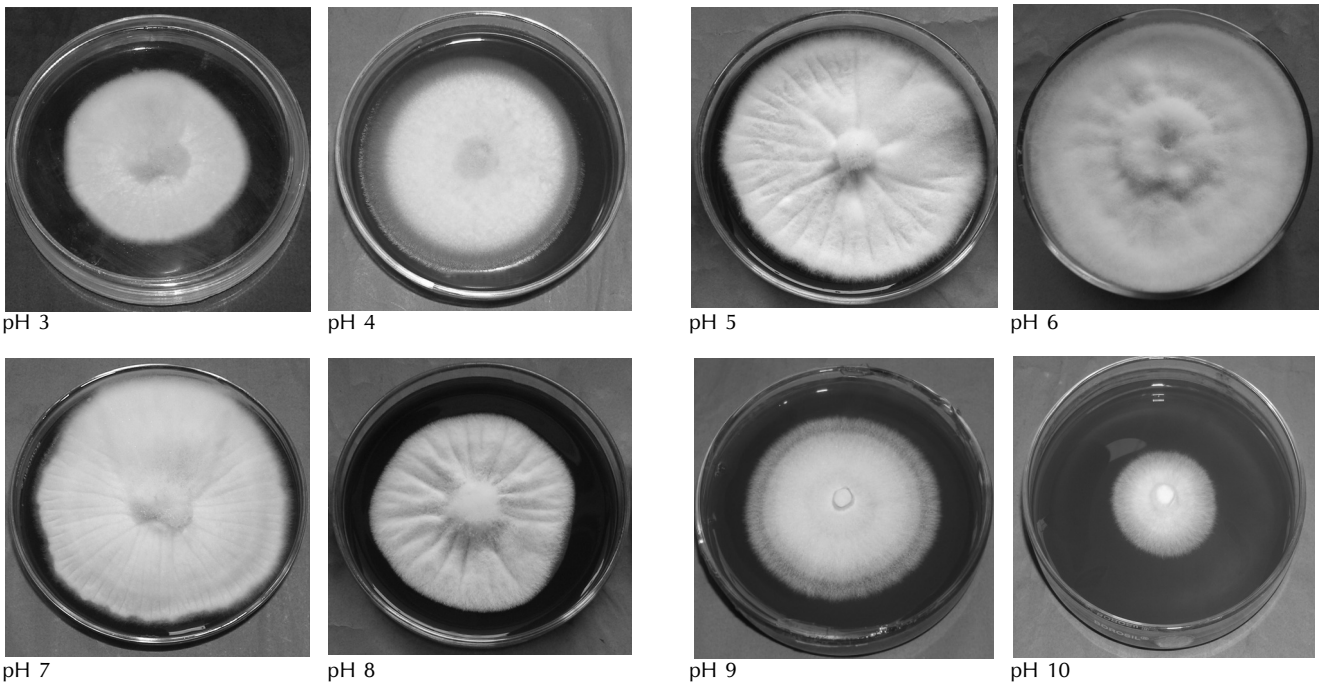
Media and temperatures for growth

Of the five different media and temperatures, tested for the growth of *C. sinensis* varied significantly from each other. However, maximum and minimum growth of the fungus was 44.93mm and 32.60mm respectively, on SDAY and CDA on 12th day (Table 1, Fig. 4).The growth on PDA was next to SDAY and significantly higher than those of OMA and MEA.SDAY was found to be the best suited medium for the



SDAY CDA 15ºC 25ºC

Figure 4: Radial growth (max. and min.) on media SDAY and CDA and temperature at 15ºC and 25ºC on SDAY, respectively



pH 3 pH 4 pH 5 pH 6

pH 7 pH 8 pH 9 pH 10

Figure 5: Radial growth of *Cordyceps sinensis* at different pH on SDAY medium

mycelial growth of *C. sinensis* and therefore, selected as a basal medium.

The radial growth of anamorph of *C. variabilis* was very slow in different culture media. Radial growth of the fungus reached to 7.0mm after two weeks only on SDAY (Hodge *et al.,* 1998). However, colony diameter of 60.1mm on PDA medium of *C. pruinosa* in four weeks incubation was recorded (Min Woong, 2004). The variations in growth recorded by these workers might be due to different species of the fungus tested for growth. However in case of the temperature studies, the maximum (44.87mm) and minimum (34.27mm) growth of the fungus was recorded at 15ºC and 25ºC, respectively (Table 2, Fig. 4). There was significant decline in growth below and above 15ºC. But the higher temperatures showed slow growth as compared to the low temperatures. The 15ºC temperature was found to be optimal for mycelial growth which decreased on both at lower and higher temperatures, are in agreement with the findings of Gwang-po Kim, (2000a); Dong and Yao, (2005).

pH for Growth

Radial growth of *C. sinensis* was studied at different pH 3-10 maintained in SDAY media (Table 3, Fig. 5) and it was found that the pH 6 supported the maximum radial growth of 44.93 mm. The minimum growth obtained at pH 3 and pH 10 were at par with each other. There was decline in growth below pH 5 and above pH 6. In general, the initial pH of solid medium for *Cordyceps* was good in the range of pH 5.0-7.0 (Gwangpo Kim 2000a; Lee *et al.,* 2000; Min-Woong, 2004; Das *et al.,* 2005). Yin and Qin (2009) and Jiu *et al.* (2009) optimized the initial pH of 6.0 and 7.0 for submerged cultivation of *Cordyceps sinensis* and for the culturing of *Cordyceps sinensis* in liquid culture, respectively

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