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ISOLATION, IDENTIFICATION AND PROXIMATE ANALYSIS OF THE MYSTERIOUS CATERPILLER FUNGUS *Ophiocordycepsneovolkiana* FROM KASARGOD DISTRICT

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Abstract: *The Cordyceps genus includesentomopathogenic fungi on arthropods. Many of the members of this genus like Ophiocordycepssinensis have remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous, sexual, immunological systems, besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities.With the increasing interest on Ophiocordyceps sp. both for mycology and medicine, research is necessary to obtain an overview on the cultivation and nutritive values of this mushroom.Purposive sampling surveys were carried out for the occurrence of fruiting bodies of Ophiocordyceps in coastal sandy areas of Kasargod district of Kerala during June- September. Isolation and identification of Ophiocordyceps was done. The molecular characterization of the fungus was carried out by ITS sequencing to identify at species level and sequence analysis showed homology with Ophiocordycepsneovolkiana having 98 per cent identity.The nutritional parameters like carbohydrate, protein, fibre, moisture, ash and ascorbic acid content of Ophiocordyceps was done by proximate analysis of fruiting bodies.This medicinal mushroom is a rich source of dietaryfibre, minerals and ascorbic acid, but it is having lower protein and carbohydrate content.*

Key words: *Isolation, Proximate analysis, Ophiocordycepsneovolkiana, Kasargod.*

Introduction

Cordyceps sp. is an entomopathogenic fungus on arthropods which on germination, kills and mummifies their larva, and then grows from the body of the host. This fungus is known in Tibet as the "winter worm, summer grass" or "Caterpillar fungus" (Yartsagunbu) (Kaszak, 2014). Many fungi belonging to *Cordyceps* have been used as food and herbal medicine in Asia (Kuo *et al.*, 2005). This fungus was known as *Cordyceps sinensis* until 2007, but molecular analysis resulted in the naming of a new family *Ophiocordycipitaceae* and the transfer of several *Cordyceps* species to *Ophiocordyceps*. Based on the molecular phylogenetic study, Sung *et al.* (2007) separated the mega genus *Cordyceps* into four genera, *viz. Cordyceps*, *Ophiocordyceps*, *Metacordyceps*, and *Elaphocordyceps*. As a result, *C. sinensis* was transferred to *Ophiocordyceps*. Due to the strict parasitism and special geographic environment in which it grows, the output of natural *Ophiocordyceps* cannot meet market demand and thus its price is unusually high. In the Indian market, this fungus is valued as much as 5 lakh rupees/Kg in recent years. Most of the bioactive compounds of *Ophiocordyceps* sp. have been exploited for use in traditional and modern ethnomedicine, for treatment of various diseases like cancer, diarrhea, headache and muscle pain. The distribution of this fungus is cosmopolitan but mostly confined to high Himalayan mountains in China, Tibet, Nepal, and India, at an altitude of 3000 to 5000 meters (Sharma, 2004). From the recent surveys conducted in coastal sandy areas of Kasargod district, it is observed that *Ophiocordyceps* sp. is attacking the coconut root grubs (*Leucopholis coneophora* Burm), which is an endemic pest of coconut plantations. The fungus parasitizes the root grubs and kills them to form a sclerotium and eventually the fruiting body of fungus emerges from the head region of the grub during SW monsoon months. The abundance of the fruiting body of this fungus is very high in cashew and coconut plantations during July - August months.

The isolation, identification, and analysis of the nutritional value of this fungus were not done in Kerala until now. In this context, the present study was to isolate, identify and assess the nutritional status of *Ophiocordyceps* sp. from Kasargod district of Kerala.

Materials and methods

1. **Survey for the collection of *Ophiocordyceps*sp.**

   Purposive sampling surveys were conducted in three locations of coastal sandy tracts of Kasargod district during June to September months of 2017 and fruiting bodies of *Ophiocordyceps* sp. were collected. The three locations selected were Instructional Farm, College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicode.

2. **Isolation and identification of *Ophiocordyceps*sp.**

   Isolation of fungus was done from different parts of the fungal structure *viz*., sclerotia, stipe, and stroma of both fresh and dry specimens by following standard tissue culture technique. The samples were washed under running tap water and cut into small bits using a sterile blade. From all the three parts, sclerotia, stipe, and stroma inner and outer portions of tissues were taken for isolation. These tissues were cut into small pieces, and those were disinfected with mercuric chloride (0.1%) for one minute. After three washings using sterilized distilled water, the samples were placed on solidified Potato Dextrose Agar (PDA) medium aseptically in sterile Petri dishes under a laminar air flow chamber. After incubation at room temperature (26 ± 2°C), the fungal growth from second to sixth days of incubation was subsequently subcultured to solidified PDA in sterile Petri dishes. Purification of isolates was done and then periodic subculturing and maintenance of the isolates was done in PDA slants under refrigerated condition at 4°C for further studies. Identification of the isolate was done at species level by molecular characterization by DNA barcoding using universal primers of ITS.

3. **Proximate analysis of *Ophiocordyceps* sp.**

   Analysis of proximate constituents like carbohydrate, protein, fibre content, total minerals, moisture, ash content and vitamin C was conducted using the collected sporocarps and mycelialgrowth obtained on the potato dextrose broth. The samples were dried and powdered for the proximate analysis. The moisture content was estimated on wet weight basis whereas the rest of the parameters were analysed on dry weight basis following standard methods of analysis.

Determination of carbohydrate

Carbohydrate was determined by Anthrone method (Hedge and Hofreiter, 1962), in which 100mg of the powdered sample was weighed and transferred to a boiling tube. Hydrolyzationof the sample was done with 5ml 2.5N hydrochloric acid by keeping in boiling water bath for 3 hrs and then cooled to room temperature. Then neutralization was done with solid sodium carbonate until the effervescence ceases. The volume was made up to 100ml and centrifugation was done. The supernatant was collected and 1 ml aliquot was taken for analysis. The standards were prepared by taking 0, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standard. Zero served as the blank. The volume was made up to 1ml in all tubes including the sample tubes by adding distilled water. Then 4ml of the Anthrone reagent was added to all the tubes and heated for 8 min in a boiling water bath. Samples were cooled rapidly and read the green to dark green colour at 630 nm using spectrophotometer. A standard graph was drawn by plotting concentration on x axis and absorbance on y axis. From the graph the amount of carbohydrate was calculated in the sample tube using the following formula.

$$\text{Amount of carbohydrate/100mg of sample} = \frac{\text{mg of glucose}}{\text{Volume of test sample}} \times 100$$

Determination of protein

Protein estimation was performed by Lowry's method (Lowry *et al.*, 1951).

Extraction of protein

500mg of the sample was weighed and ground it well with a pestle and mortar in 5-10 ml of buffer. This was centrifuged and supernatant was used for the for protein estimation.

Estimation of protein

Into a series of test tubes, 0.2, 0.4, 0.6, 0.8 and 1 ml of the working standards were pipette out and then 0.1ml and 0.2 ml of the sample extracts were also pipette out in two other test tubes. The volume was made upto 1 ml in all the test tubes. A tube with 1 ml water served as the blank. 5 ml of alkaline Cu solution were added to each tube, mixed well and allowed to stand for 10 min. Then 0.5ml of Folinciocalteaure agent was added mixed well and incubated at room temperature in dark for 30 min, for the development of blue colouration. The readings were taken at 660nm using a spectrophotometer. A standard graph was drawn, and the amount of protein was calculated and expressed as mg/g of the sample.

Determination of crude fibre content

Estimation of crude fibre was done by following methods described by Maynard (1970) and Misra *et al.* (1975). Two gram of ground material was extracted with ether or petroleum ether to remove the fat. After extraction 2g of dried the sample was boiled with 200ml of sulphuric acid for 30 min with bumping chips. Filtration was done through muslin and washed with boiling water until washings were no longer acidic. Then boiling was done with 200ml of sodium hydroxide solution for 30 min. again filtration was done through muslin cloth and washed with 25ml of boiling 1.25% sulphuric acid, three 50ml portion of water and 25ml alcohol. The residue was transferred to preweighedashing dish (W1) and then dried for 2 hrs at 13 ± 2^{0}C. The dish was cooled in a desiccator and weighed (W2). Then ignition was done for 30min at 600± 15^{0}C, cooled in a desiccator and reweighed (W3).The per cent of crude fibre in ground sample was calculated by using the formula,

$$\% \text{ crude fibre} = \frac{\text{Loss in weight on ignition}}{\text{Weight of sample}} \times 100$$

Loss in weight on ignition = (W2-W1) - (W3-W1)

Determination of moisture

Five grams of the sample (W1) was taken in a preweighed crucible and dried in hot air oven until a constant weight was obtained (W2). The difference between the initial and final weight gives the moisture content, which is then converted and expressed in percentage.

$$\% \text{ moisture content} = \frac{W1 - W2}{W1} \times 100$$

Determination of total ash

One gram of the sample was transferred to a weighed silica crucible (W1). It was heated on a Bunsen burner at low flame and when the substrate charred the crucible was transferred to a muffle furnace. It was heated at 500 to 550ºC for about 2 hours till a white coloured ash was obtained. It was then cooled in a desiccator and weighed (W2). The difference between the weights gives the ash content, which is then converted and expressed in percentage.

$$\% \text{ of Ash} = \frac{W2 - W1}{\text{Weight of the sam}} \times 100$$

Determination of Ascorbic acid

Volumetric method was followed for Ascorbic acid estimation (Harris and Ray, 1935). Initially 5 ml of working standard solution was pipette out into a 100 ml conical flask. 10 ml of 4 % oxalic acid was added and titrated against the 2, 6-dichlorophenol indophenol dye (V1 ml). End point was determined by the appearance of pink colour, which persists for a few minutes. The amount of dye consumed was calculated as the amount of ascorbic acid. Then the sample (0.5-5 g depending on the sample) was extracted in 4 % oxalic acid and made up to 100ml and centrifugation was done. 5 ml of the

supernatant was pipette out and10 ml of 4% oxalic acid was added to it. Then titration was done against 2, 6- dichlorophenolindophenoldye. (V2ml)

$$\text{Amount of ascorbic acid mg/100g sample} = \frac{0.5\text{mg}}{\text{V1ml}} \times \frac{\text{V2ml}}{5\text{ ml}} \times \frac{100\text{ml}}{\text{Weight of the sample}} \times 100$$

Result and discussion

1. **Survey for the collection of *Ophiocordyceps* sp.**

Purposive sampling surveys were conducted at Instructional Farm, College of Agriculture, Padannakkad, Valiyaparamba area of Nileshwar and Regional Agricultural Research Station, Pilicodeof Kasargod district during May to September months of 2017 and fruiting bodies of *Ophiocordyceps* sp. were collected. The fruiting bodies were found to be emerged after summer showers usually during 2nd week of May or after onset of monsoon showers during 1st week of June (figure 1). Kumar and Aparna (2014) reported the occurrence of *Cordyceps* sp. on coconut root grub atcoastal sandy tracts of Kasargod district. Maximum number of fruiting body was found at Instructional Farm, College of Agriculture, Padannakkad under cashew plants (figure 3).

2. **Isolation and identification of *Ophiocordyceps* sp.**

Samples collected from the survey were isolated, purified and maintained on PDA by periodic sub culturing. The fungus was isolated on PDA medium andthe isolate obtained after pure culturing was named as CD1.Amin *et al.* (2008) got similar observations, in which the mycelial growth of *Cordycepssinensis* was maximumon PDA medium and optimum pH was 8.5 to 9.5 and temperature was 20^0-25^{0}C for growth. Arora *et al.* also used PDA for isolation of *Cordycepssinensis* (Berk.)Sacc. from Uttarakhand**.** The mycelia appeared initially white, later turning to creamish white to salmon colour and the underside of the plate being light brown (Figure 2). Later on numerous thread like pinkish synnemata appeared in culture representing the anamorphic stage *Hirsutella*sp. The isolate completed the full growth in a 9×9 cm2 petri plate by 55 days after inoculation. The anamorph produced similar type of conidia as produced in the field. The molecular characterization of the fungus was carried out by ITS sequencing to identify at species level. Sequence analysis and nucleotide homology of the fungus wasanalysed through the BLAST programme of NCBI (http:// ncbi.nlm.nhm.gov/blast). Sequence analysis of the *Ophiocordyceps* culture showed homology with *Ophiocordycepsneovolkiana* strain KC 1having 98 per cent identitywhich was reported by Sangeetha, C. and Krishnamoorthy, A.S. in 2015 from Tamilnadu. Kumar and Aparna (2014) found out that *Cordyceps* is found to be naturally occurring as an entomopathogenicfungi on coconut root grub (*Leucopholisconeophora* BURM) in Kasargod district and the species is capable of suppressing tumour cells and inhibiting the proliferation of lung cancer (adenocarcinoma) cells.

Figure 1: Fruiting body of *Ophiocordycepsneovolkiana* collected from

Figure 2: Culture plate of *Ophiocordycepsneovolkiana*

Figure 3: fruiting bodies collected from field

3. Proximate analysis of *Ophiocordyceps*sp.

The proximate analysis of the fruiting bodies and cultured mycelium of the fungus *Ophiocordycepsneovolkiana* was done as per the procedure mentioned above and the results are given in the Table -1. The carbohydrate content was found to be 5% and 9.7% in fruiting body and mycelium respectively. Protein content was estimated using Lowry's method and found to be 2.9% and 1.75% in fruiting body and mycelium respectively. Moisture content was 76.8% in fruiting body and 27.6 % in mycelium which is very less than the moisture content of fruiting body. The ash content was found to be 1.96% and 2.1% in fruiting body and mycelium respectively. The ascorbic acid content was found to be 3.27% in fruiting body and 2.5% in mycelium. The crude fibre content obtained in this study was 16.8 % in fruiting body and 25% inmycelium, which is relatively higher and therefore this medicinal mushroom is a rich source of dietary fibre. The ash amount was determined to characterize content of the total minerals, which is high in this fungus. Minerals are required for the growth, development, repair and maintenance of the body. Presence of ascorbic acid improves the nutritional value giving an additional significance to its medicinal properties.The proximate analysis of *Cordycepsmilitaris*was done by Pathania*et al.* (2015),collected from North West Himalaya and reported that itis rich incarbohydrate, protein, vitamins and fibre content.

Due to recent advancements in pharmaceutical biotechniques, it is possible to isolate bioactive compounds from *Ophiocordyceps* and make it available in powder as well as in capsular form. In 2012, Patel and Goyal reported that*Cordyceps* and its product have remarkable clinical health effects including action on hepatic, renal, cardiovascular, respiratory, nervous, sexual, immunological systems, besides having anti-cancer, anti-oxidant, anti-inflammatory and anti-microbial activities. With the increasing interest on *Ophiocordyceps*, both for mycology and medicine, research is necessary to obtain an overview about the cultivation of this mushroom. So keeping in view the importance of this valuble fungus, studies on its ecological niches, mycological perspectives should be performed in future for effective utilization and conservation of this fungus.

Table 1: Nutritional status of *Ophiocordycepsneovolkiana*

SL.NO	PARAMETER	VALUE IN %*	
		Fruiting body	Mycelium
1	Carbohydrate	5	9.7
2	Protein	2.9	1.75
3	Crude Fibre	16.8	25
4	Moisture	76.8	27.6
5	Ash content(Total minerals)	1.96	2.1
6	Ascorbic Acid(Vitamin C)	3.27	2.5

*Mean of 4 replications

Conclusion

The present study to isolate, identify and assess the nutritional status of *Ophiocordyceps* sp. from the Kasargod district of Kerala got a booming result. Due to its rare occurrence in nature, especially in Kerala, its successful isolation and identification is an exceptional work in this field. The fruiting body development is not found to be a successful process; thereforeproduction of mycelia by *in vitro* culture is the only promising alternative for utilization of this fungus. Since *Ophiocordyceps* is attacking on the coconut root grubs (*Leucopholisconeophora*Burm), role of this fungus as a biocontrol agent also need further research. Keeping in view about the anti-cancer property of this valuable fungus, studies on its ecological niches, mycological perspectives should be performed in future for effective utilization and conservation of this fungus.

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