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CHARACTERIZATION AND OPTIMIZATION OF CULTURE CONDITIONS OF CORDYCEPS SINENSIS

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Abstract

In previous few decades, many organisms inhabiting both terrestrial as well as aquatic habitats have been target of researchers for various uses in medicines. Both lower plants as well as higher plants have been analyzed widely and are being used for the production of secondary metabolites. *Cordyceps sinensis* is a medicinal mushroom put into endangered category due to its uncontrolled exploitation and harvesting from its natural habitats. The fungus has a rare modified nucleoside Cordycepin, being used in many medicinal formulations. The authors have collected and characterized various isolates of *Cordyceps* from high altitude regions and optimized its culture conditions for its use in suspension culture and mycelia production. It was observed that the standard pH conditions and input of nitrogen compounds in the potato dextrose medium elicit mycelia production rate in suspension cultures. Though the high level of associated microbial flora makes it difficult in identification and purification of the cultures, but use of elicitors in suspension cultures for specific metabolites could be of great use on economical scale.

Key words*: Cordyceps sinensis*, peptone, nucleoside, suspension culture

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Introduction

Cordyceps sinensis is a medicinal mushroom and found on high altitude regions in Himalayas. It is commonly called as Yarshagumba and Kida Jadi in Uttarakhand. It belongs to Ascomycetes and is reported to have more than 400 species (Chamberlain, 1996). It is a parasite mainly on the larvae of lepidopteran insects. The fungus has two stages, asexual phase (anamorph) and sexual phase called as telomorph. The telomorph phase develops only after the infection to insect eggs and larvae (Gi Ho Sung et al., 2007). The accumulated compounds collected in larval body have significant medicinal potential and local peoples gather them from under the ice cover after the death of larvae (Grey and Barker, 1993). Since the mushroom is very rare and though many new techniques have been developed for its artificial cultures, peoples are harvesting it randomly from hilly areas. Many local workers take risk to collect the herb from high altitudes and put their life in danger. The fungal sticks are sold in the black market @rate of Rs 20 lakhs/kg. Due to the random and uncontrolled exploitation from native habitats, its existence has been put under threat. The governments have banned its harvesting and provide limited license to local villagers only so that the biodiversity of fungus could be protected.

Many therapeutic Applications of *Cordyceps* have been discovered especially in Chinese traditional medicines for the treatment of respiratory, pulmonary, renal, liver, heart related disorders, sexual weakness and hyper-lipidemia (Shin et al., 2001 & 2003). Many medicines are in use for the treatment of various

immune disorders and cancer therapy (Nakamura et al., 1999 & 2003; Bok et al., 1999). The *Cordyceps* extracts are thought to be possible remedy for weakness, fatigue and is suggested to be used as rejuvenator (Creadon & Dam, 1996). Regular use of medicinal preparations is recommended to increase the resistance potential against microbial infections, colds and flues. It is also said to improve the homeostasis helping in kidney related disorders and increase cellular ATP levels (Guowei, 2001; Parcell et al., 2004).

It has been reported that many fungal derived simple and protein-bound polysaccharides exert a significant improvement of immune function which is being considered as possible mechanisms of anticancer activity (Wasser, 2002; Smith et al., 2002). Some of the unique nucleosides found in *Cordyceps* are Cordycepin, dideoxyadenosine, hydroxyethyladenosine etc that could be targeted, in elicitor- induced cell cultures to improve the medicinal potentials of fungal mycelia.

MATERIAL AND METHODS:-

Collection and Identification

Fungal sticks were harvested from the Lata and Dolma Vidhan Panchayats, District; Chamoli, Uttarakhand in the month of June to September, 2012. *Cordyceps sinensis* specimens had a less plump caterpillar body with a long (an expanded head at the stroma tip. These samples represented mature telomorph phase of *Cordyceps sinensis* (about 4.0-8.0 cm in height). The sticks were dark brown to black stroma. All freshly collected *C. sinensis* specimens were washed thoroughly in running water with gentle brushing, sterilized in 0.1% mercuric chloride for 10 min for surface sterilization and washed three times with sterile water. After proper surface sterilization and cleaning, *C. sinensis* specimens were kept frozen during transportation and storage prior to further processing.

Culture Optimization

Axenic cultures were prepared and optimized in artificial potato dextrose agar (PDA) plates. Broad range antibacterial antibiotics were used to avoid unnecessary bacterial growth. Serial dilution method was applied to get pure cultures of *Cordyceps sinensis*. The starter cultures were then transferred to nutrient broth containing 10 g/L peptone and 30 g/L glucose at pH 6.0, on a rotary shaker at 120 rpm for 96 h at 28^{o}C (Holliday et al., 2004). The fungal mycelia were collected and stored at −80^{o}C. Suspension cultures were obtained in 250 ml Erlenmeyer flask containing 50 ml of medium on a rotary shaker at 25^{0}C and 150 rpm-300 rpm.

Morphological studies

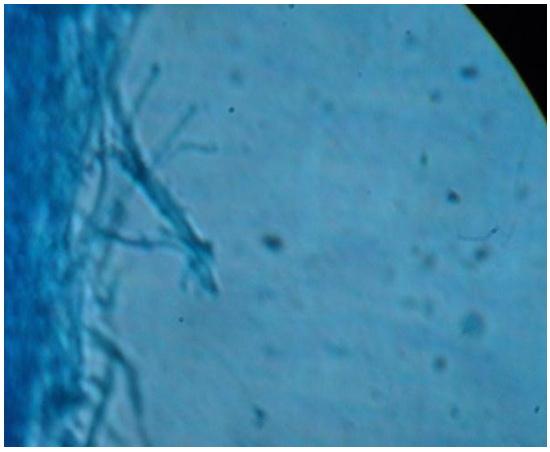
Mycelia growth on different pH and agitation intensity was observed for analyzing the growth characteristics. The images were taken using an Optical microscope and a Nikon 1600 (7.1 Megapixel) photographic camera. Various experimental samples were analyzed to get the optimum mycelia growth. Nitrogen sources such as $NaNO_3$, KNO_3 and Peptone in various concentrations were standardized to get maximum mycelia production. Optimum pH and temperature were also recorded for the characterization of the fungus and monitored under optical microscope.

RESULTS AND DISCUSSION:-

Microscopic characterization of *Cordyceps sinensis*

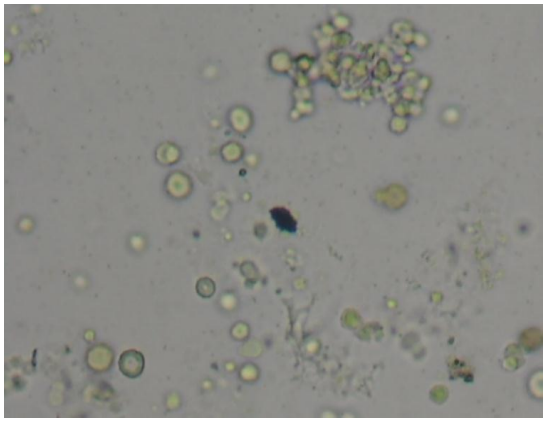
It was observed in the transverse section of stroma that perithecia were elliptical and embedded at the surface of the fertile portion of stroma. Central part was observed to be full with hyphae with clefts present between the hyphae. Larval body in the transverse section was seen to be 23–85 mm thick with bristles 20–40 mm in length on the surface. It was filled with whitish hyphae, with L-shaped stripes. Yellowish brown pitchy stipes with abundant hyphae and bristles were observed at the surface of larval body (figure 1 & 2).

Figure 1: Stroma and sclerotium of a *Cordyceps sinensis* along with the larval body with 8 pairs of feet on abdomen (4 pairs in the centre)



(a)

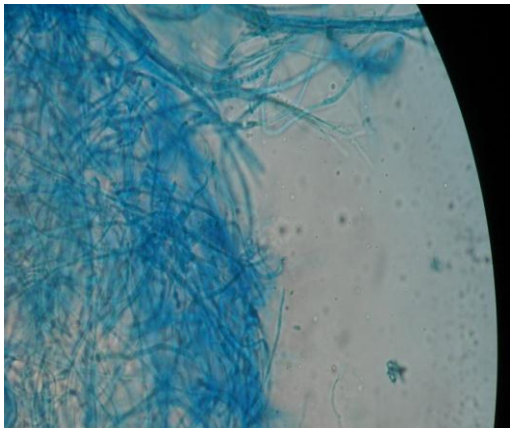
(b)



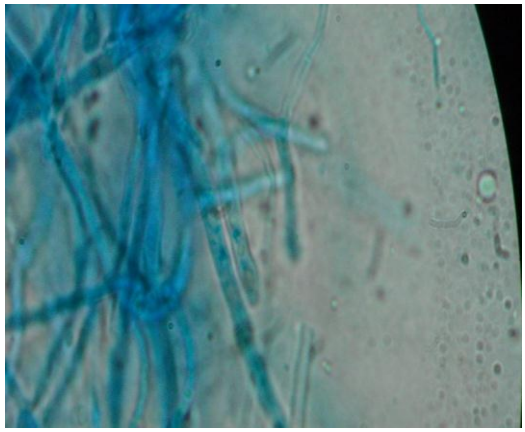
(c)

Figure 2 (a) Fungal mycelia and bristles emerging from the larval body (cross section), (b) fungal hyphae and (c) spores of *Cordyceps sinensis*

It was observed that the fungal cultures could be well prepared with slow growth rate in potato dextrose agar medium. Purified axenic cultures were further used in liquid medium. It was evident from the study that high agitation proved to be better for the maximum mycelia production at optimized rate. Liberated ascospores were observed in the cultures prepared from telomorph stage of fungus (figure 2). It was clear from the results that pH 6-7 was suitable for mycelia growth in suspension cultures with pH 6.4 (figure 3 & 4).

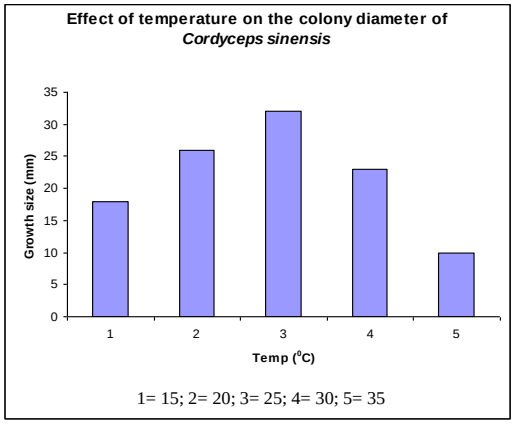


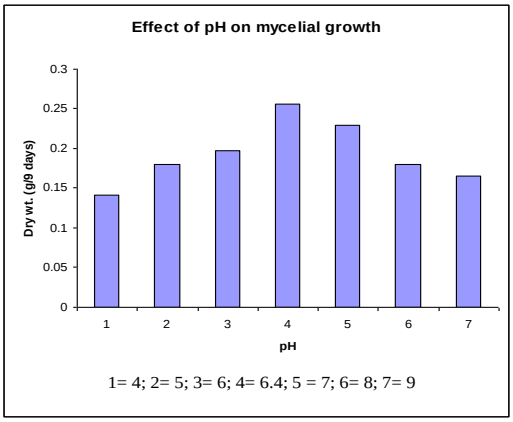
(a)

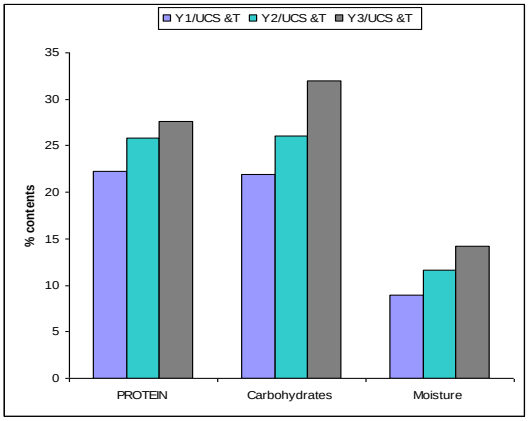


(b)

Figure 3: Dense fungal mycelia growth in peptone enriched medium (PDB).







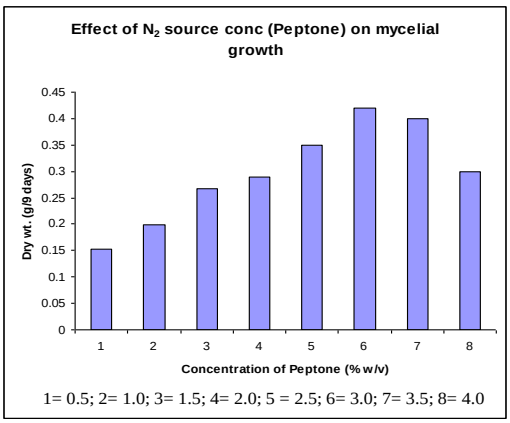


Figure: 4: Growth optimizations and biochemical analysis in *Cordyceps sinensis*.

Since the fungus grows in low temperature regions, we analyzed optimum temperature for liquid culture isolates. It was clear from the results (temperature range 15-35^{0}C) that the maximum mycelia growth was recorded at 25$\pm$2^{0}C. Peptone enriched medium was found to be the best for fungal growth. Variable range of peptone was used in terms of % w/v and 3 gram peptone was observed to be the best. All isolates were studied for its protein, carbohydrate and moisture contents. Protein was extracted and estimated by using Lowry method and Carbohydrate by standard Anthrone method. The % protein, carbohydrate and moisture contents with respect to the dry weight were 23-28%, 19-25% and 7-15% respectively (figure 4).

Being an endangered category fungus, it is necessary to analyze the optimized culture conditions for *Cordyceps sinensis* and its possible uses in suspension cultures for metabolite regeneration. It was evident from the results that fungal mycelia could be regenerated and harvested in lab conditions with suitable media enrichments and improving culture conditions. It was observed and reported by other authors previously that telomorph phase cannot be obtained in liquid cultures which is essential for Cordycepin production. It needs larval host to complete the life cycle. But the presence of ascospores in large quantity in suspension cultures could be helpful in regenerating the asexual stage of fungus on partially decomposed paddy raw material. The harvested mycelia could have a possible use in artificial inoculation in other insect classes like *Bombyx mori* and artificial fungal sticks with nucleoside contents might be produced. Various biological elicitors can also be used for the enhancement of metabolites in cell cultures.

Cordyceps is also called as a medicinal magic due to its wide use in different formulations. Some peoples also call it as 'Himalayan viyagra' for its application in boosting sexual power. Its use in treatment of cancer and respiratory disorders made it highly valuable (Bok *et al*, 1999). It needs more attraction towards its conservation in the native habitats for possible uses in future. Many researchers are attempting to get suitable somatic hybrids of *Cordyceps* and interrelated genera for maximum socioeconomic benefits. It will not only help in protecting the species in its natural habitats but will open a new era of metabolite production.

Conclusions

It is evident from the results that Cordyceps sinensis can be grown in vitro in optimized liquid culture media with suitable pH, temperature and nitrogen source. Peptone enriched medium was found to be most suitable for the mycelia production. Harvested mycelia can be grown for ascospore production with partially decomposed paddy straw to generate asexual anamorph phase. The harvested mycelia can be used for protoplast preparations for somatic hybrid generation. It is highly needful to conserve the diversity of fungi in native locations for possible uses in future.

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