

Exploring the Potential of *Cordyceps militaris* Cultivation on Agricultural Waste for Sustainability and Economic Development

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ABSTRACT

Cordyceps militaris is utilized for several medicinal properties and is one of the high-priced mushrooms in the market. Cordycepin, the compound produce by the fungus, has a potent antibacterial action against the majority of bacterial species. The production of *Cordyceps militaris* is limited and to meet the market demand, the production needs to be increased. The insights into cultivation techniques of the fungus are must to enhance its production. Thus, a study was conducted to evaluate the growth of fungus on seven different mediums and agriculture waste-based substrates. Amongst all the tested media, maximum radial growth was observed after 16 days in CZYA (6.8 cm) and OMA (6.7 cm). Use of Brown rice as a substrate resulted in maximum numbers of fruiting body production, in minimum number of days compared to other substrates.

Keywords: *Cordyceps militaris*, agricultural wastes, substrate, biopesticide

Cordyceps sp. is one of the medicinally important mushrooms, which has remarkable pharmacological activity and still requires a lot of research to make it available for mankind. Genus *Cordyceps* have a history of medicinal use spanning millennia in parts of Asia (Gu *et al.*, 2007). It has been regarded as a cornerstone of traditional Chinese medicine for centuries; that has a number of several medicinal effects (Holliday *et al.*, 2005). It has several bioactive compounds like cordycepin, adenosine, p-Hydroxybenzoic acid, mannitol, polyunsaturated fatty acids, -tocopherol, and -(1 → 3)-D-glucan (Liu *et al.*, 2014; Wen *et al.*, 2017; Smiderle *et al.*, 2014; Elkhateeb *et al.*, 2019). It is also used to treat immune defects and treats modern cancer treatments as a supplement along with conditions including respiratory and pulmonary diseases, renal, liver, cardiovascular, hyposexual and hyperlipidemic diseases (Holliday and

Cleaver, 2008; Khan *et al.*, 2010) and is known to have anti-inflammatory effect (Smiderle *et al.*, 2014). Developing new cost-effective techniques capable of cultivating the species can contribute to producing enough quantities of bioactive compounds such as cordycepin and which also may lower the cost of this expensive medicinal mushroom.

Of all *Cordyceps* species, *C. militaris* is the only species that has been successfully cultivated and most intensively studied. The majority of *Cordyceps* products on the market are obtained from cultivated *C. militaris* fruiting bodies. Regarding the artificial cultivation of *C. militaris*, the main challenge is the growth rate of mushroom mycelium on the industrial scale preparation by making the cultivation time shorter together with increasing the mycelial density. This fungus is characterized by a very low growth rate

when grown in a solid agar medium for inoculum preparation during the cultivation process. Thus, there is an enormous need to increase the growth rate of this fungus on a solid medium to reduce the time of the inoculum preparation stage.

Growing fruiting bodies of *C. militaris* using cereal grains is more popular because of its easy operation, and fruit body products from this model prevail in the market. However, raw materials for fruit body production have become a problem with the development of the cultivation industry. China produces around 4000 tonnes of dry fruit bodies each year, using at least 5,000 tonnes of grain substrates in the process. Cereal materials are more expensive and get contaminated more easily than agricultural trash. For a long time, agricultural wastes have been used in mushroom growing, showing good adaptability and economic effectiveness (Panjikaran and Mathew, 2013; Yang *et al.*, 2016). However, there are only a few reports about their application in the cultivation of *C. militaris*. It is not only beneficial for industrial production to alleviate the pressure of human demand, but will also protect the limited natural resources of the fungus for sustainable utilization.

MATERIAL AND METHODS

Effect of different media on growth of *Cordyceps militaris*

Mycelial discs of 5 mm diameter were obtained from the periphery of actively growing mycelial colonies with a pre-sterilized cork borer under aseptic conditions, to be used as inoculum in solid media for physiological studies. Seven solid media *i.e.* Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar plus Yeast Extract (SDAY), Malt Yeast Agar (MYA), Malt Extract Agar (MEA), Oat Meal Agar (OMA), Czpek Yeast Extract Agar (CZYA) and V-8 Juice Agar (V8) were evaluated during the present studies. The Petri plates having different solid media were incubated for 10 days at 25 °C in an incubator (BOD) under dark conditions. Vegetative growth of mycelium in the solid media was measured by taking the diameter of the colony in two directions at right angles. Three replicates of each medium were used and average values were taken for comparison of growth in different media (Sehgal and Sagar, 2006).

Liquid Spawn Preparation

For cultivation of fruiting bodies, liquid spawn of fungus was used. Due to the homogeneous distribution

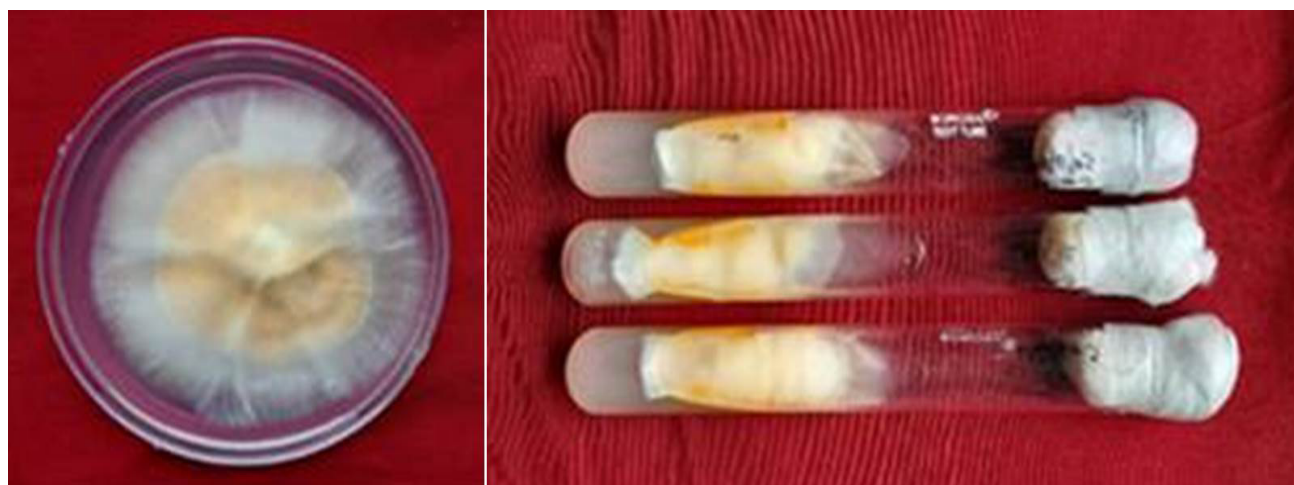


Fig. 1. Pure culture of *C. militaris* on (A) Petri plate (B) Test tubes

of inoculum in the substrate, liquid spawn takes less time to colonize the substrate than solid spawn. GPBY (Glucose Peptone Beef and Yeast Extract) liquid medium was used to produce liquid spawn. A 250 mL flask containing 100 mL GPBY broth was inoculated with 2-3 pieces of 5 mm mycelium bits of the fungus and incubated at 25°C with shaking at 120 rpm. After 7 days of growth, inoculum was ready showing hyphal bodies of *C. militaris*. The spawn culture was diluted with 4 volumes of sterilized distilled water and utilized to inoculate the substrate for the formation of fruit bodies (Lin *et al.*, 2016).

Agriculture Waste Substrates preparation

Wheat straw, sawdust, poultry litter, rice straw, waste papers, tea waste and coco peat were used in the experiment as substrate and were locally collected from nearby areas of Pantnagar, UK, India. All substrates were cleaned 2-3 times in tap water and dried overnight at room temperature (25°C). The next day, the substrates were sterilized in an autoclave for 30 minutes at 15 psi pressure. Substrates comprising 20 g of dry material were pressed uniformly into 700 ml bottles. 20 mL of nutritional liquid containing 20 g L⁻¹ glucose and 5 g L⁻¹ peptone were used to wet the substrate. Brown Rice media, including 20 g of Brown rice and 20 mL of nutritional liquid per bottle, was used as a control. The bottles were sterilized in an autoclave at 15 psi pressure for 30 minutes, using autoclavable lids with 5 mm holes plugged with cotton plugs. After autoclaving, the substrate was allowed to cool to ambient temperature before being inoculated.

Inoculation and Fruit Body Cultivation

Inside the laminar airflow chamber, inoculation was done maintaining aseptic conditions. With the aid of a 10 mL syringe, the liquid spawn culture of *C. militaris* kept in a sterile flask was injected equally into each container at the rate of 10 mL per bottle.

In the incubator, all culture bottles were placed in the dark at a temperature of 20°C with a relative humidity of 60–70 per cent. The incubator was lit with fluorescent light at an intensity of 500–800 lux for 10 hours per day once mycelia had grown over the medium surface. Meanwhile, regular ventilation for 30 minutes was performed on a daily basis. The cotton plugs were removed when the aerial and surface vegetative hyphae became orange-yellow to allow for greater air exchange. The culture conditions were kept the same until the fruit bodies grew spherical and coated in spores. The Fresh weight of fruit bodies was measured shortly after harvesting following maturity. The number of days taken for spawn run, primordia to appear in the media, maturation time, stromata length and number of stromata per bottle was counted along with characteristics of fruiting bodies. Three replicates were carried out for each treatment as per the method given by Lin *et al.*, (2016).

RESULTS AND DISCUSSIONS

Effect of different media on the Radial growth of *C. militaris*

Seven solid media i.e. Potato Dextrose Agar (PDA), Sabouraud Dextrose Agar plus Yeast Extract (SDAY), Malt Yeast Agar (MYA), Malt Extract Agar (MEA), Oat Meal Agar (OMA), Czpek Yeast Extract Agar (CZYA) and V-8 Juice Agar (V8) were tested for the cultural characteristics and radial growth of *C. militaris*. The linear growth was recorded up to 16 days of the incubation period. Table 1 reveals that maximum radial growth (6.8 cm) was observed on 16 days in CZYA and OMA (6.7 cm) and differ significantly from the rest of the media tested. Minimum growth of 2.0 cm was observed in MEA, which was statistically at par with V8 (2.1 cm) and MYA (2.3 cm).

The results obtained agreed with Shrestha *et al.* (2006) who examined the characteristic growth

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Table 1. Effect of different media on growth of *Cordyceps militaris*

S No.	Media	Average Colony diameter (cm)	Colony characters			
			Mycelial Density	Texture	Margin	Zonation
1	PDA	4.3	++	Semi-Cottony	Smooth	Absent
2	V8	2.2	++	Semi-Cottony	Smooth	Absent
3	MYA	2.3	++	Flat	Serrate	Present
4	MEA	2.0	++	Semi-Cottony	Smooth	Absent
5	OMA	6.7	+++	Cottony	Serrate	Present
6	CZYA	6.8	+++	Cottony	Filiform	Absent
7	SDAY	6.1	++	Flat	Serrate	Present
	SEm±	0.104				
	CD at 5%	0.318				
	CV	4.130				

(-) extremely poor; (+) poor; (++) moderate; (+++) abundant

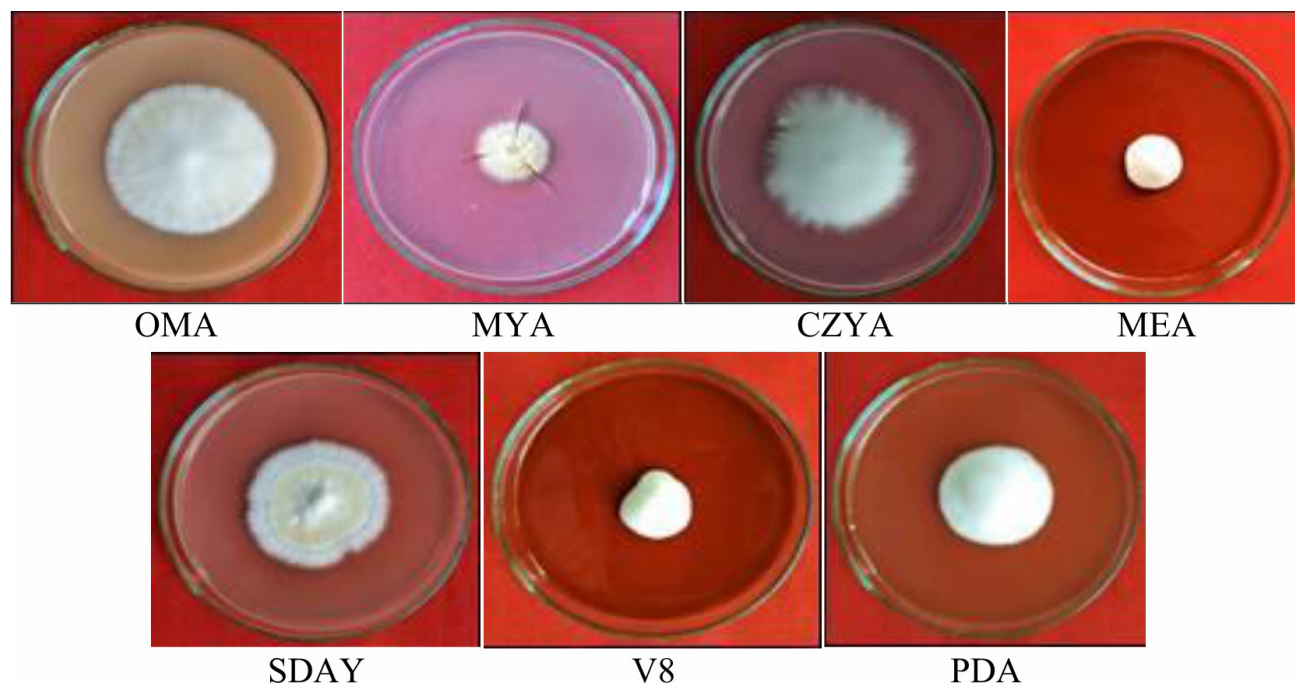


Fig. 2. Growth of pure culture of *C. militaris* on different solid media under dark condition at 25 °C

pattern of *C. militaris* isolates and found abundant growth and mycelial density of fungus isolates produced in nutritionally rich media like Czapek Yeast Extract agar (CZYA), suggesting that these media may fulfill all the requirements for vegetative growth

of the fungus. Czapek Yeast Extract agar (CZYA) and OMA media supported the best growth of the fungus. It might be due to the presence of extra supplements of mineral salts in CZYA media like $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, KCl , $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and K_2HPO_4 ,

which helps to enhance the growth of fungus as confirmed by previous studies (Kang *et al.*, 2014 and Li *et al.*, 2004). In OMA, the presence of a high amount of carbohydrates, proteins and lipids as compared to other media may be responsible for the fast-radial growth of the fungus.

Growth of *Cordyceps militaris* on Agriculture waste substrate

The days taken for spawn run on different substrates ranged from 4.33 days to 12 days. Minimum days required for spawn run was in T6 (waste paper) i.e. 4.33 days which was significantly better over the rest of the treatments followed by T8 (brown rice) and T7 (poultry litter) i.e. 6.33 and 6.66 days, respectively. While the maximum days required for spawn run in treatment T4 (rice straw) was 12 days thus, this treatment was inferior in comparison to the former. All the treatments showed white cottony growth of fungus except T3 (Tea waste) and T5 (Wheat straw), which showed flat growth with round depressions on the mycelium mat along with yellowish pigmentation. The results aligned with Lin *et al.* (2016) who observed fastest spawn run and fruiting body formation in *Flammulina velutipes* in brown rice compared to other substrates like Cottonseed Shells (CS), Corn Cob Particles (CCP) and Italian Poplar Sawdust (IPS). Different rates of spawn run may be due to differences in C/N ratio, porosity and composition of the substrate. All solid waste substrates have features in common, primarily their macromolecular structure, which is normally composed of cellulose, lignocellulose, pectin or other polysaccharides. The natural material served both, as a nutrient source and as support for fungal growth. Higher biodegradability helps the fungi to spread over the substrate, leading to better growth and sporulation. Several of the agricultural wastes; such as rice husk, wheat bran and tea waste supported excellent sporulation in entomopathogen *B. bassiana*, showing

potentiality to be used as a substrate of choice for mass production of this fungus (Mishra *et al.*, 2016). Different rates of spawn run may be due to differences in C/N ratio, porosity and composition of the substrate.

Days Required for Primordia Formation

Among eight treatments only three substrates (Tea waste, poultry litter and brown rice) were able to produce pin heads. Among these three treatments, T3 (Tea waste) recorded the highest number of days (19.33 days) for pinhead formation followed by 15.66 days in T7 (Poultry litter) and 11.33 days in T8 (Brown rice). The number of primordia was more in the case of control (Brown rice), which was yellowish in colour as compared to Tea waste and Poultry litter, both of which were orange in colour. In the case of Poultry litter, the shape of pinheads was found abnormal (irregular and blunt) while Brown rice and Tea waste produced normal pinheads (clavate to cylindrical). The results were similar to that of Lin *et al.* (2016) who reported that *Flammulina velutipes* (SS) produced primordia in 12 days in Brown rice. The reason for no fruiting body in the other substrates may be non optimum C/N ratio as Kontogiannatos *et al.* (2021) reported that *Cordyceps militaris* could not use cellulose as the sole carbon source and has an optimal C/N ratio for growth that is 12.7:1 for the production of fruiting bodies. From the above discussion, it can be concluded that by use of the different supplements, we can modify the composition, C/N ratio and porosity etc. of agricultural waste substrates so that they can be used for fruiting body production.

Days required for maturation of fruiting bodies

The number of days required for maturation of fruiting bodies revealed that the minimum days required in T8 (brown rice) i.e. 48.33 days. Maximum days observed in T3 (Tea waste) i.e. 59.66 days,

Table 2. Effect of different substrates on growth and development of *Cordyceps militaris*

Tr. No.	Treatments	Days required to complete spawn run	Days required for Primordia Occurrence	Fruit body mature time in days	Total number of stromata per bottle	Stroma length (cm)	Yield (Fresh weight) per bottle in gm	Biological efficiency (%)	Fruit body appearance
T ₁	Saw dust	10	0.00	0.00	0.00	0.00	0.00	0.00	-
T ₂	Coco peat	09	0.00	0.00	0.00	0.00	0.00	0.00	-
T ₃	Tea waste	8.66	19.33±0.57	59.66±0.57	64.66±5.03	4.60±0.4	10.00±0.43	50	Orange-yellow, clavate to cylindrical in shape. Initially having narrow pointed tips which later becomes round
T ₄	Rice straw	12	0.00	0.00	0.00	0.00	0.00	0.00	-
T ₅	Wheat straw	9.33	0.00	0.00	0.00	0.00	0.00	0.00	-
T ₆	Waste paper	4.33	0.00	0.00	0.00	0.00	0.00	0.00	-
T ₇	Poultry litter	6.66	15.66±0.57	53.66±0.57	2.33±0.57	7.76±0.20	2.83±0.15	14.15	Orange-yellow, cylindrical in shape having irregular and blunt tips.
T ₈	Brown rice (Control)	6.33	11.33±0.57	48.33±0.57	55.66±3.51	5.53±0.30	15.03±0.20	75.15	Orange-yellow in colour, Clavate to cylindrical in shape having narrow pointed tips

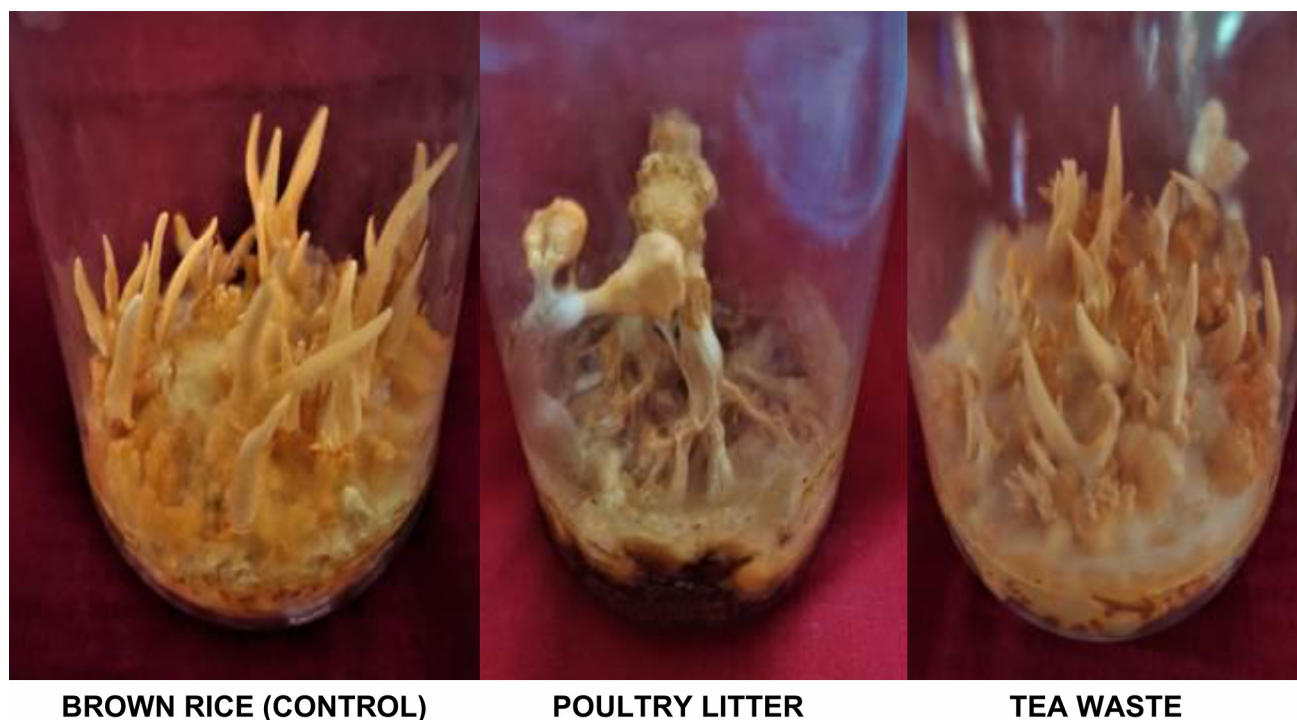


Fig. 3. Fruiting bodies of *Cordyceps militaris* on different substrates

which was inferior in comparison to the other treatments followed by T7 (poultry litter) i.e. 53.66 days. The earlier reports are in conformity that stromata of *Cordyceps militaris* are usually produced over a period of 35–70 days (Zhang and Liu, 1997; Yue 2010; Du *et al.* 2010).

Yield of fruiting bodies in different agricultural waste

Among all the three treatments, the maximum number of stromata per bottle were produced in Tea waste (64.66) followed by Brown rice (43.66). Poultry litter showed a smaller number of stromata (2.33). But in the case of length of stroma, Poultry litter showed the longest stroma (7.76 cm) as compared to Brown rice (5.53 cm) and Tea waste (4.60 cm). The overall yield of the fruiting bodies (fresh weight per bottle) was maximum in Brown rice (15.03 g) followed by Tea waste (10.00 g) and least in Poultry litters (2.83 g). Similarly, maximum Biological efficiency was in Brown rice (75.15%)

followed by Tea waste (50%) and Poultry litter (14.15%). Thus, overall Brown rice medium showed better results in *Cordyceps* production as compared to other treatments used during the study.

Brown rice produced orange-yellow coloured fruiting bodies, which were clavate to cylindrical in shape having narrow pointed tips. Fruiting bodies produced by Tea waste were orange in colour having cylindrical to clavate body with initially narrow pointed tips which later becomes round on maturity. While fruiting bodies formed by Poultry litter were Orange-yellow in colour, cylindrical in shape with irregular and blunt tips. The results aligned with Lin *et al.* (2016) who reported that the length of the stromata of *C. militaris* in Cottonseed Shells (CS), Corn Cob Particles (CCP) and rice media in the range of 5–6 cm. Similar results were obtained by Sharma *et al.* (2024). The yield obtained was in accordance with Wu *et al.* (1996) who obtained 25 g of fresh fruit bodies of *Cordyceps militaris* from 50 g of rice medium, while Zhang and Liu (1997) reported a

biological transformation rate of 61 per cent on rice, 58–59 per cent on millet, and rice-tussah media, and 42 per cent on maize. Production of 18.0 g of stromata (fresh wt.) has been obtained from 20 g of rice (Lin *et al.* 2006). Also, nearly 9 g of dry stromata (equivalent to about 68 g of fresh wt.) was produced from 60 g of brown rice supplemented with 10 g of silkworm pupae (Sung *et al.* 2006).

CONCLUSIONS

Amongst the tested mediums, maximum radial growth was observed after 16 days in CZYA (6.8 cm) and OMA (6.7 cm). On evaluating waste substrates minimum time for spawn run was in T6 (waste paper) i.e. 4.33 days. Minimum time was required for maturation of fruiting bodies in T8 (brown rice) i.e. 48.33 days. The overall yield of the fruiting bodies (fresh weight per bottle) on Brown rice (15.03 g) was maximum followed by Tea waste (10.00 g) and least in Poultry litters (2.83 g). These results can pave the way to explore various waste substrates for fruiting body production of *C. militaris*. Their easy availability and low cost will lead to a decrease in the cost of production for the farmers and help in the environmental protection. Hence, more focus should be needed in this area of research as very few studies are there on this topic.

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