Original Research Article

Selection of the best performing media for the growth and development of *Cordyceps militaris* mycelia

ABSTRACT:

Since ages, the genus *Cordyceps* has been used as a medicinal fungus for augmenting endurance, longevity and vitality. This fungus commands a high commercial value due to scarcity in its availability and high global market demand. In the present investigations different defined media *viz*, agar, optimised nutrient broth, chitosan + malt extract agar, chitosan + agar + optimised nutrient broth, Kenknights and Munnaiers, malt extract agar, malt extract agar + optimised nutrient broth, oat meal agar, rice extract agar, rice extract agar + optimised nutrient broth and rose bengal agar have been used to identify the optimal media supporting the growth of *Cordyceps militaris* mycelia. After 14-days of culture, chitosan + agar + optimised nutrient broth, Kenknights and Munnaiers media, oat meal agar media and rice extract agar media recorded better mycelial growth, in comparison to agar, that was used as control. Maximal mycelial growth was recorded on Kenknights and Munnaiers media, with a 20% higher growth than the control. Even after 21 days of culture, Kenknights and Munnaiers media recorded best growth characteristics with a 25% higher mycelial growth. It was also recorded that the mycelial growth rate on Kenknights and Munnaiers media was highest on the 14th day, in comparison to 7th and 21st day after inoculation. Thus, our results provide valuable scientific information towards *in-vitro* culture of *Cordyceps* with optimal growth dynamics, for sustaining market demand of this important mushroom.

1. INTRODUCTION:

Mushroom species can grow under varied environmental conditions and their species distribution is determined by their requirement of specific temperature, humidity, food, daylight conditions etc. In the last decade the global demand for mushrooms has increased significantly due to their edible value as well as their medicinal uses. Thus, mushroom cultivation has emerged as a profitable business with substantial scope for filling up the demand to supply gap. There are several factors like shelf life, cropping time, spawn quality, mycelial run rate, etc. that needs to be investigated for improving the quality of mushroom grown by farmers.

Cordyceps spp. is one of the most important species amongst mushrooms and its existence is known since 2000 BC. This important mushroom is also known by various vernacular names such as

keedajadi, yarsagumba, keera ghas, himalayan viagra, and sanjevani-booti *etc* (Tuli et al. 2014). Some of the earliest mentions of the medicinal usage of this mushroom have been documented in Sowa-Rigpa system of medicine from 1439-1475. The usage of *Cordyceps* can be traced back to 15th century, wherein it is included as medicinal herb in Chinese Materia Medica. *Cordyceps* is used for revitalization of various systems of the body and is considered as one of the oldest herbs used traditionally.

Cordyceps belongs to phylum Ascomycota classified in the order *Hypocreales,* and it produces spores internally, inside a sac-like structure called ascus (Tuli et al. 2014). This entomopathogenic (insect parasitizing) fungus is primarily found on the head of the soil-dwelling larva of *Hepialus armoricanus* Oberthur (Lepidoptera) moths but is infrequently recorded growing on other moth species also (Kai et al. 2013). *Cordyceps* grows parasitically on insects and small arthropods (lepidopteron larvae, pupae of insects, ants, spiders, etc), wherein it infects and kills them by using their bodies as substrate for growth (Chen et al. 2024). The infection of *Cordyceps* starts when the conidia of *Cordyceps* secrete certain hydrolytic enzymes like lipase, proteases, chitinase, leading to dissolution of chitin and also providing it nutrition (Tuli et al. 2014, Wang et al. 2005).

Since ancient times, people in China, Tibet, Nepal and India have taken *Cordyceps* spp. to help their bodies adjust to the harsh conditions of the mountains, including low temperature, low oxygen levels, as well as high atmospheric pressure (Tuli et al. 2014, Kai et al. 2013). It is considered as an adaptogen, which helps in simulation of immune system and has the ability to reduce fatigue (Kai et al. 2013). *Cordyceps* could be extensively used in modern system of medicine and is of great interest in medicinal applications. This mushroom is an abundant source of natural products with several biological uses (Jiapeng et al, 2024). *Cordyceps* contains compounds that have the potential to improve oxygen uptake and endurance, as well as have anti-inflammatory and antioxidant effects (Kai et al. 2013). It is increasingly becoming popular as a dietary supplement, particularly for athletes and fitness enthusiasts. Since, it enhances physical performance and increases energy. Cordycepin, the active component, may facilitate human sperm capacitation by adenosine receptor-mediated signalling pathways (Shan et al 2024).

Fruiting bodies of *Cordyceps* contain fat soluble vitamins (vitamin A and vitamin E) and water-soluble vitamins (vitamin B2, vitamin B3 and vitamin C). *Cordyceps* has also been reported to provide relief from symptoms of chronic bronchitis, asthma, influenza a viral infection, inflammation, kidney disorders and other respiratory disease/infections (Ohta et al. 2007, Wu et al. 2024). Numerous natural dietary extracts from *Cordyceps* have been demonstrated to be effective in the induction of cell cycle arrest and apoptosis in cancer cells, under *in vitro* conditions (Jin et al. 2008, Gong et al. 2024). It can be used to improve blood lipid (fat) levels and treating arrhythmia (irregular heart beat). Thus, *Cordyceps* can be extensively used in modern system of medicine and is of great interest for its nutraceutical applications. It is a medicinal mushroom which have abundant source of natural product with several biological usages. In view of this, the current study was planned to evaluate various defined media for inducing optimal in-vitro culture of *Cordyceps.* Such a study will lead to augmenting the supply of *Cordyceps* and also for enhancing the production of secondary metabolites in this important mushroom.

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1.1. Natural Habitat:

Cordyceps militaris is found in tropical regions and humid temperate climates broadly distributed throughout Europe, North America, East and Southeast Asia. *Cordyceps militaris* has similar medicinal properties as *Cordyceps sinensis* which majorly grows at high altitude Himalayan Alpine Meadows. *Cordyceps sinensis* is mostly found in China's, Sichuan, Qinghai, Yunnan, Gansu and Tibet Autonomous Region provinces. *Cordyceps* spp. has been found in Asia, North America and Europe, mostly in countries such as China, Nepal, Bhutan, Thailand, Vietnam, Korea and Japan (Tuli et al. 2014). *Cordyceps sinensis* grows particularly in some specific habitats and often inhabits grassland soil at an elevation of 3500 to 5000 m (Bharat et al. 2020). It is naturally distributed in the high-altitude grassland of Nepal, Bhutan, India and Tibetan plateau of China. This species is also found in some areas of the Uttarakhand (Garhwal and Kumaon regions), Sikkim, Arunachal Pradesh Himalayas viz. Chamoli District, Pithoragarh District, Suthol, Kanol, Munsiyari-Darchula region, Niti-Mana Valley, Chiplakot, Ultapara, Brahmkot, Najari, Nagnidhura, Laspa region, Lachen, Lachung and Gnathang (Bharat et al. 2020, Tuli et al. 2014, Caplins et al. 2017, Negi et al. 2012). The collection and gathering of this fungus by many of the Bhotiya communities has exploded and have emerged as a lucrative yet high-risk livelihood strategy (Caplins et al. 2017).

Due to parasitic activity of moth's larva in the arid alpine environmental condition and over-exploitation of *Ophiocordyceps sinensis,* now called *Cordyceps sinensis,* have led to risk toward unnatural extinction (Krishna et al. 2024). *Cordyceps sinensis* is designated as vulnerable species as per IUCN Red list of threatened species in 2019 and China biodiversity red list 2018 (Wang et al. 2021). Vulnerable species means a species at high risk of human-caused (un-natural) extinction without further human interventions (Mace et al. 2008).

Cordyceps militaris can be cultured on both liquid and solid media. *Cordyceps militaris* is considered as a promising substitute of *Cordyceps sinensis* because of similar medicinal properties, chemical capacities and have higher levels of certain bioactive components (Chou et al. 2024). In recent years, its artificial cultivation is in high demand, where production cost and cultivation period can be optimised. Several researchers have reported that *Cordyceps militaris* have shorter cultivation time and lower production costs under artificial cultivation (Chou et al. 2024). After the outbreak of COVID, consumption of naturally available *Cordyceps* has increased and is expected to grow from $1.2 billion in 2023 to $1.23 billion in 2024, at a compound annual growth rate (CAGR) of 9.8%. (*Cordyceps sinensis* Global Market Report 2024) The continuous increase in global demand poses a serious ill-effect on the climate and biodiversity (habitat destruction) of the area in the hills. So, there is an urgent need for its artificial cultivation.

2. MATERIAL AND METHODS:

2.1 Culture collection and maintenance:

Pure culture of *Cordyceps militaris* (DMR 1163) was obtained from Directorate of Mushroom Research (DMR), Solan, Himanchal Pradesh, which was cultured and maintained on Malt Extract Agar medium.

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2.2 Preparation of culture media:

The following table describes in detail the composition of different culture media used in the current study. The media was sterilized before use.

Contents of media (in gm)	Agar	Agar + ONB	Chitosan + Agar + ONB	MEA	MEA + ONB	Chitosan + MEA	REA	REA + ONB	OMA	RBA	K&M
Agar	20	20	20	20	20	20	20	20	12.5	15	15
Chitosan	-	-	10	-	-	10	-	-	-		
Dextrose	-	30	30	20	50	20	-	30	-	10	1
Magnesium Sulphate	-	0.75	0.75		0.75		-	0.75	-	0.5	0.1
Malt Extract	-	-	-	20	20	20	-	-	-	-	-
Mono Potassium Phosphate	-	-	-	-	-	-	-	-	-	1	-
Oat Meal	-	-	-	-	-	-	-	-	60	-	-
Papaic Digest of Soyabean Meal	-	-	-	-	-	-	-	-	-	5	-
Peptone	-	7.5	7.5	1	8.5	1	-	7.5	-	-	-
Potassium Chloride	-	-	-	-	-	-	-	-	-	-	0.1
potassium Dihydrogen Phosphate	-	1.5	1.5	-	1.5	-	-	1.5	-	-	0.1
Rose Bengal	-	-	-	-	-	-	-	-	-	0.05	-
Sodium Nitrate	-	-	-	-	-	-	-	-	-	-	0.1
Tri Ammonium Citrate	-	1.5	1.5	-	1.5	-	-	1.5	-	-	-
Vitamin B1	-	0.075	0.075	-	0.075	-	-	0.075	-	-	-
Vitamin B12	-	0.015	0.015	-	0.015	-	-	0.015	-	-	-
White Rice Extract	-	-	-	-	-	-	20	20	-	-	-
Yeast Extract	-	4.5	4.5	-	4.5	-	-	4.5	-	-	-
D. D. Water (in L)	1	1	1	1	1	1	1	1	1	1	1

All the Eleven media were prepared as per above mentioned composition. Where, Agar, Agar + ONB (Optimised Nutrient Broth), Chitosan + Agar + ONB, MEA (Malt Extract Agar), MEA (Malt Extract Agar) + ONB, Chitosan + MEA, REA (Rice Extract Agar), REA + ONB, OMA (Oat Meal Agar), RBA (Rose Bengal Agar), K&M (Kenknights and Munnaiers), gm (grams), L (litre)

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2.3 Inoculation and Incubation:

The culture slants obtained, were used to prepare mother culture petri-plate of internal diameter of 85 mm and volume of 25 ml. Further these mother culture petri-plate were used to inoculate eleven different media (in four replicates). For the inoculation, cork-borer of 5 mm size was used to punch agar plug with mycelial mat. Which was pasted invertedly on the media by the help of the inoculation needle. These petri-plates were sealed by parafilm tape and were placed in the incubator maintained at 18℃ for further reading of mycelial parameters.

2.4 Measurement of Radial Growth, Mycelial Growth/Day and Mycelial density:

Mycelial growth was determined with the help of a digital vernier calipers, the diameter of the radial growth of mycelium was marked and noted on 7^{th}, 14^{th} and 21^{st} day at the drawn quadrant axis of the replicates. The average mycelial growth rate is defined as the mycelial growth obtained on 7^{th}, 14^{th} and 21^{st} day divided by number of days. Mycelial density is the mycelia present over the surface of the agar (Mahadevan and Shanmugasundaram, 2018). For the mycelial density/fluffiness the following rating was used:

+ = Low; ++ = Moderate; +++ = Abundant; +++ = Abundant; ++++ =Very Abundant

2.5 pH Adjustment/Standardization:

From the above experiments, the best performing medium was identified and on the basis of the mycelial growth. Further, the selected media was prepared with different pH (4, 5, 6, 7, 8 & 9) values on agar and liquid media which supported the best radial growth, mycelial growth. The growth measurements on different pH media were taken on 7^{th}, 14^{th} and 21^{st} day after inoculation (DAI). In the liquid media the result was taken on the basis of the fresh weight, dry weight and the moisture content. The acidity and basicity of the media was maintained by 3N NaOH and 3N HCl.

2.6 Statistical analysis:

One Factor Analysis of Variance (ANOVA) was conducted for the experiments with mean significance difference ($P < 0.05$) among the treatments by the use of OPSTAT. Each value is expressed as mean and SE (n=4).

3 RESULT AND DISCUSSIONS:

In order to culture any mushroom under *in-vitro* laboratory conditions, it is required to search the best components which can increase the performance and quality of the spawn. Here, we have measured the growth of mycelium over different media for selecting the optimal media that can promote maximal growth and can reduce the time of crop/flushes. In the present study *Cordyceps militaris* was cultured successfully in the laboratory environment. In the present

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investigation different media i.e., Agar, Agar + Optimised Nutrient Broth (ONB), Chitosan + Malt Extract Agar (MEA), Chitosan + Agar + ONB, Kenknights and Munnaiers (K&M), Malt Extract Agar (MEA), Malt Extract Agar (MEA) + Optimised Nutrient Broth (ONB), Oat Meal Agar (OMA), Rice Extract Agar (REA), Rice Extract Agar + Optimised Nutrient Broth, Rose Bengal Agar (RBA) were used to identify their effect on *Cordyceps militaris* mycelial growth.

The mycelium radial growth was recorded on 7th, 14th and 21st day after inoculation. The readings were pooled from data of two independent experiments. The results showed mycelial radial growth/radial diameter of *Cordyceps militaris* on 21st day was ranked highest on Kenknight and Munnaiers media with 8.22 cm, the second highest radial growth was obtained in rice extract agar with 7.74 cm and for the third highest radial growth for Agar + optimized nutrient broth at 6.96 cm **(Graph 1)**. For the mycelial growth per day was calculated with dividing the growth with number of days. The highest growth rate/day on 21st day was recorded on Kenknight and Munnaiers media at 0.39 cm/day, followed by rice extract agar at 0.37 cm/day and agar + optimized nutrient broth at 0.33 cm/day **(Graph 2)**. It was also recorded that the growth rate on Kenknight and Munnaiers media was highest on the 14th day 0.43cm in comparison to 7th day 0.40cm and 21st day 0.39cm.

The results obtained from the liquid media experimentation recorded highest fresh weight and dry weight in rice extract medium (Fw = 4.04gm and Dw = 0.472gm), the second highest was recorded in the malt extract media (Fw = 1.24 and Dw=0.16). Least increment in fresh weight and dry weight was obtained on Kenknight and Munnaires media (Fw = 0.78 and Dw = 0.065) **(Graph 3A)**. Moisture content percentage was highest in Kenknight and Munnaiers (91.7%), second highest in rice extract media (88.34%) followed by on malt extract media (88.84%) **(Graph 3B)**.

For the mycelial density agar was the lowest (1/+), Kenknight and Munnaier was at moderate (2/++). malt extract agar, oat meal agar, rice extract agar, rice extract agar + optimized nutrient broth and rose bengal agar were abundant (3/+++) in mycelial density. Chitosan MEA, chitosan + agar +ONB and MEA+ONB were very abundant (4/++++) in mycelial density **(fig. 1)**.

The pH of a medium plays a vital role in regulating the growth characteristics as well as the biomass. Further, it can be immensely important in production of different metabolites. Metabolic activity of the cells varies with the change in pH value of the media. It should also be taken into consideration that the supportive pH value varies from media to media for the specific strain of mushrooms (Kaur et al. 2023). Also, it is noted that there is no prior data existing, on a systematic relation between biomass growth and bioactive metabolite production (Dudekula et. al 2020, Elisashvili 2012). The best performing medium (i.e., K&M, REA, MEA) on the basis of radial growth was chosen for the pH value analysis (4, 5, 6,7,8, 9) **(Graph 4 and 5)**. Here Kenknight and Munnaiers media was taken as control or standard with a pH of 6.87. Optimal growth was noted at pH 5, 6 and 7. This provides an indication that the media supports mycelium run rate in the pH range of 5-7.

4 Conclusion

The limited availability and high global market demand of this medicinal mushroom has led to its extremely high market price. Our research shows that in-comparison to malt extract agar, mycelial growth of *Cordyceps militaris* was better on Kenknights and Munnaiers media by 23% on the 14th day and 28% on the

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21st day. The comparison of average radial growth rate and mycelial growth rate per day of *Cordyceps militaris* mycelium shows that Kenknights and Munnaiers media is best in comparison to other media tested in the current studies. Our studies provide valuable information regarding the selection and standardization of defined media for inducing optimal mycelial growth of *Cordyceps militaris.*

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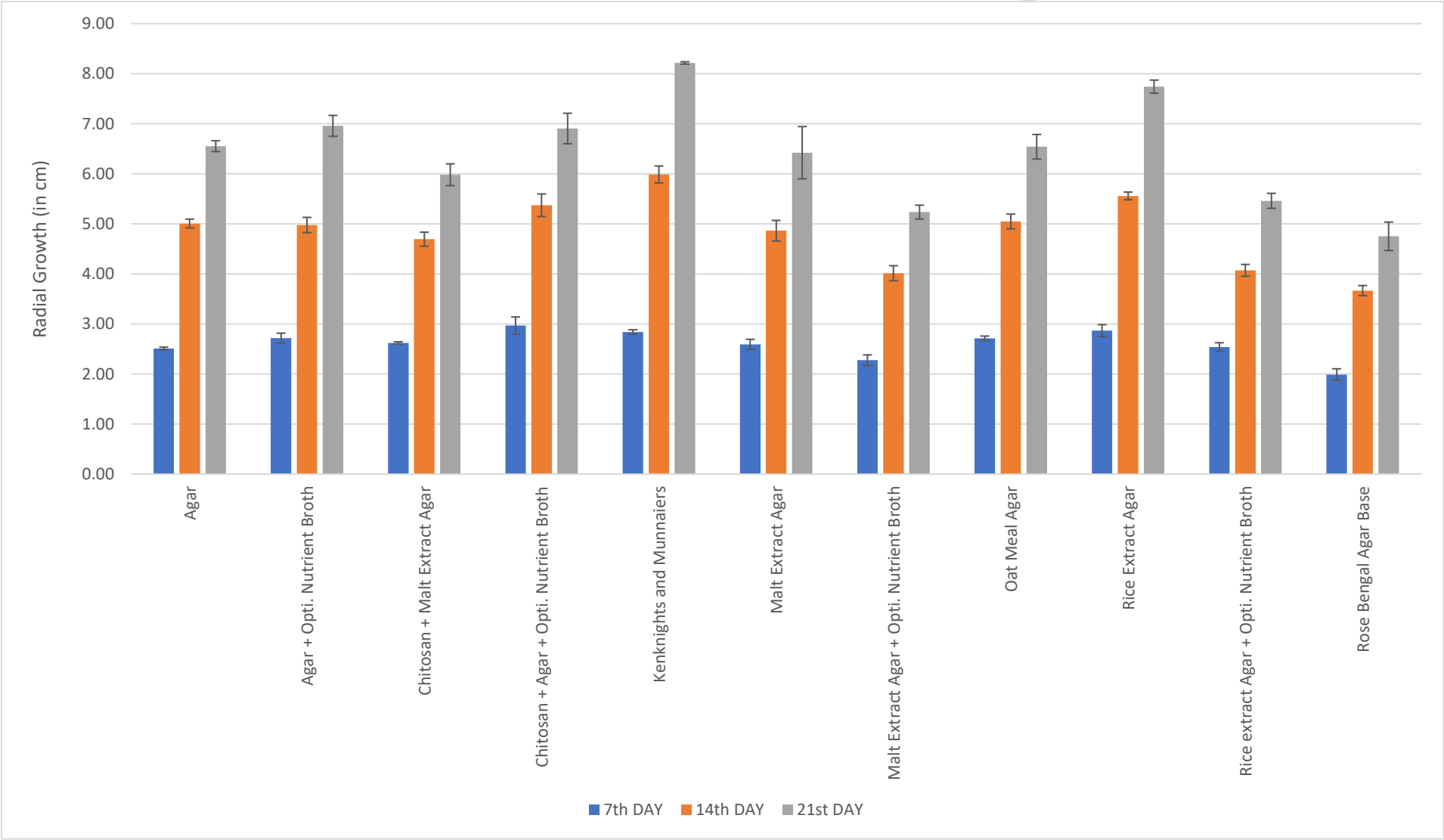
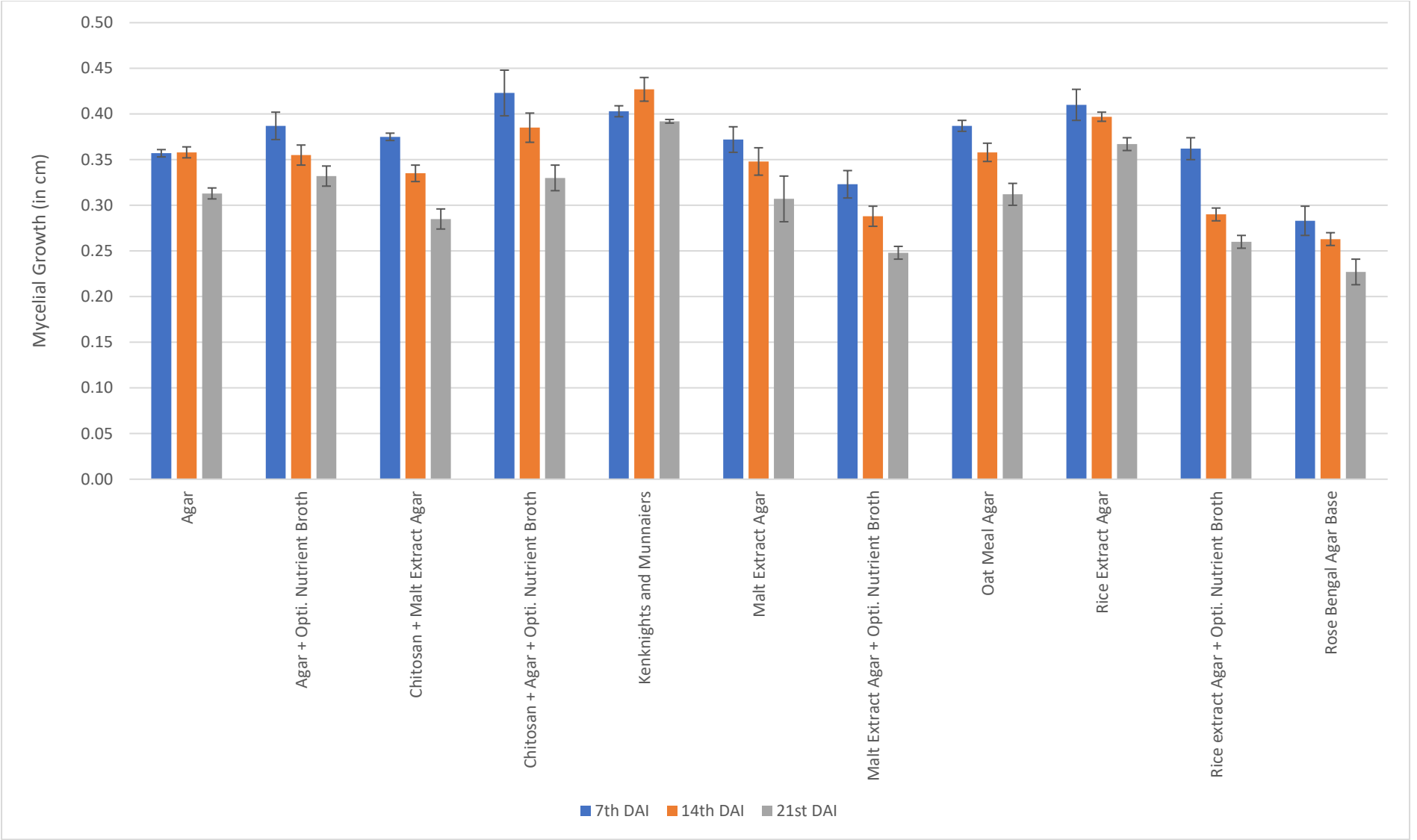
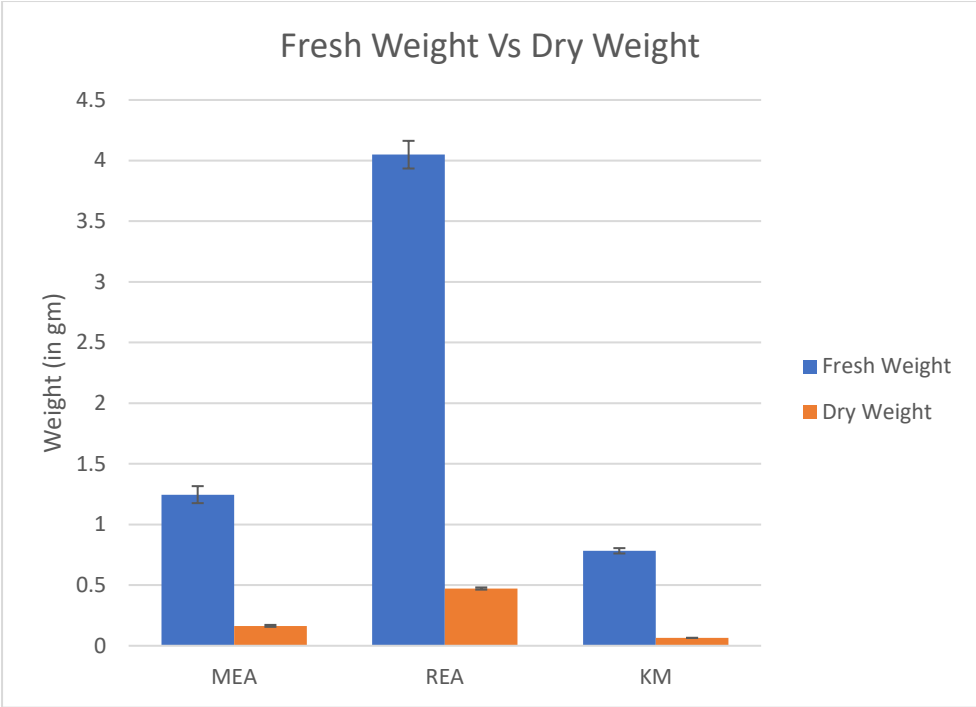
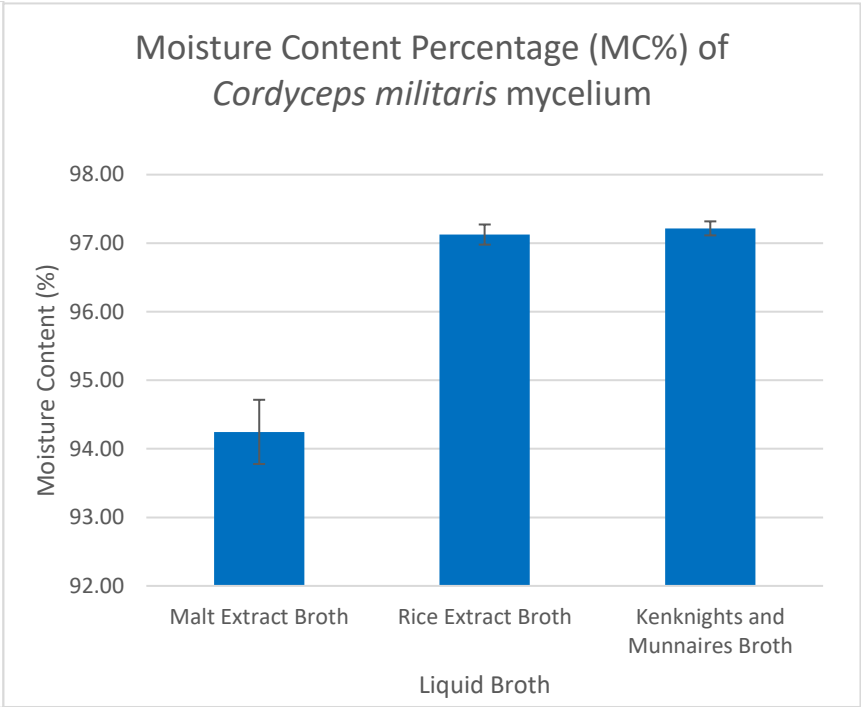


Graph 1: Average mycelium radial growth rate of *Cordyceps militaris* on different synthetic media at 7th, 14th and 21st day.



Graph 2: Average mycelial growth rate per day on different synthetic media after 7th, 14th and 21st days after inoculation (DAI).





(A) **(B)**

Graph 3: (A) Fresh Weight and Dry weight; (B) Moisture Content (in %) of *Cordyceps militaris* mycelium harvested from different liquid media.

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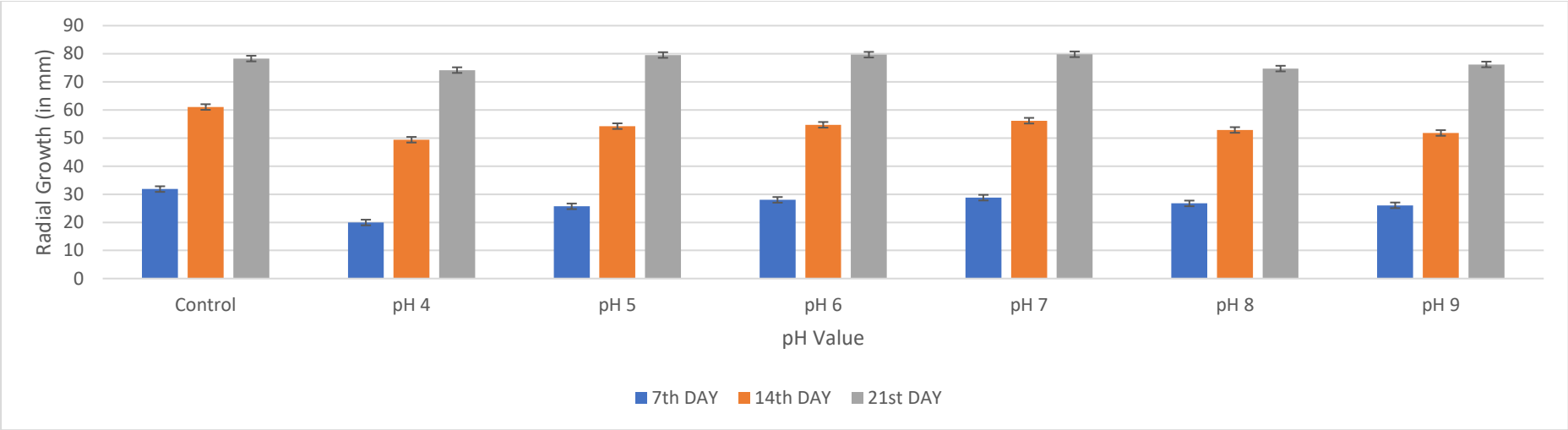
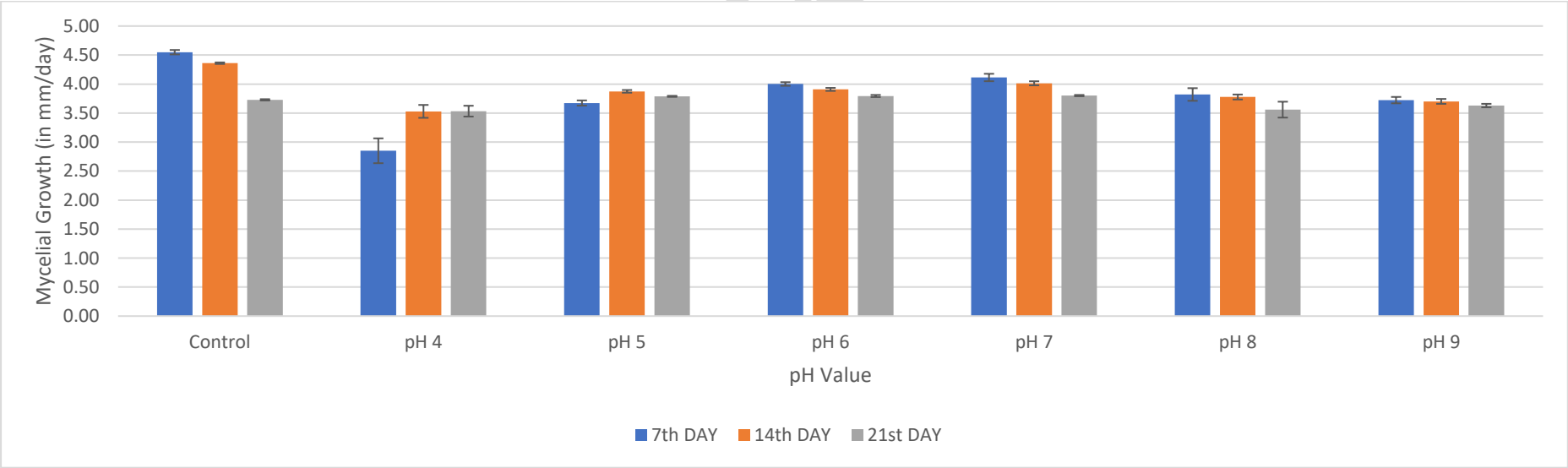


Graph 4: Radial growth of Mycelium on K&M over different pH range.



Graph 5: Mycelial growth per day on K&M over different pH range.

Agar
(+)

Agar + ONB
(++++)

Chitosan + MEA
(++++)

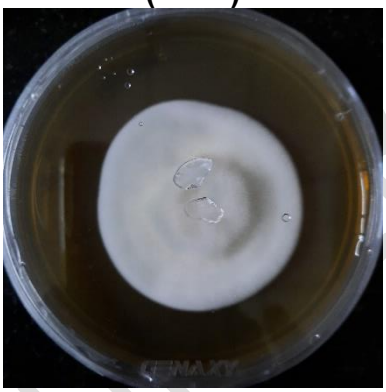
Chitosan +Agar+ ONB

(++++)

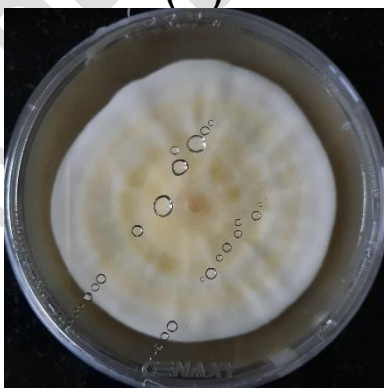
Kenknights and Munnaiers
(++)

Malt Extract Agar

(+++)

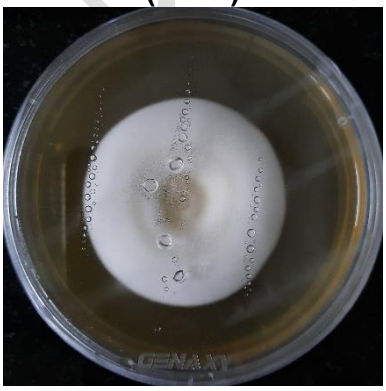


MEA + ONB
(++++)



Oat Meal Agar
(+++)

Rice Extract Agar
(+++)



REA + ONB

Rose Bengal Agar

Fig. 1: Mycelial density
+= Low
++= Moderate
+++= Abundant
++++=Very Abundant

(+++) (+++)

STATEMENTS OF DECLARATION:

Consent

Not Applicable

Ethical approval

Not Applicable

Disclaimer (Artificial intelligence)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during the writing or editing of this manuscript.

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