



e-ISSN: 2319-8753 | p-ISSN: 2347-6710

# IJRSET

International Journal of Innovative Research in  
**SCIENCE | ENGINEERING | TECHNOLOGY**



# INTERNATIONAL JOURNAL OF INNOVATIVE RESEARCH

IN SCIENCE | ENGINEERING | TECHNOLOGY

Volume 13, Issue 2, February 2024

**ISSN** INTERNATIONAL  
STANDARD  
SERIAL  
NUMBER  
INDIA

Impact Factor: 8.423

# Cultivation Methods and Antimicrobial Activity of *Calocybe indica* (Milky Mushroom)

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## ABSTRACT

*Calocybe indica*, commonly known as the milky white mushroom, is a species of edible mushroom native to India. The sturdy all-white mushrooms appear in summer after rainfall in fields and on road verges. Traditionally eaten in West Bengal, it is being grown commercially in several Indian states and other tropical countries.

KEYWORDS-*Calocybe indica*, milky white mushroom, antimicrobial, cultivation, commercial

## INTRODUCTION

*Calocybe indica* was formally described in 1974, from material collected in Kolkata. The authors—botanists R.P. Purkayastha and Aindrila Chandra—had noted it to be a popular mushroom in markets in West Bengal. They placed it in the section *Calocybe* of the genus *Calocybe*, noting that it appeared closely related to and was similar morphologically to *Calocybe gambosa*, from which it differed by having slightly larger oval spores, and a stouter mushroom.<sup>[1]</sup> Botanist A. S. Krishnamoorthy found it growing in Tamil Nadu in the mid 1990s, and its commercial production was overhauled and improved.<sup>[2]</sup> The robust mushroom is all-white in colour and has a firm consistency. Its cap is 10 to 14 centimetres (4 to 5+<sup>1</sup>/<sub>2</sub> in) across, convex initially before flattening out with age. The cuticle (skin) can be easily peeled off the cap. The crowded gills are white but gradually develop into brown with age, and the cylindrical stem is 10 centimetres (4 in) high with no ring nor volva. It has a subbulbous base, being 1.8 centimetres (<sup>3</sup>/<sub>4</sub> in) wide at the apex (top), 3.5 centimetres (1+<sup>1</sup>/<sub>2</sub> in) in the middle and 2.4 centimetres (1 in) wide at the base. The mushroom does not change colour on cutting or bruising, though old dried specimens have a buff colour. The flesh has a mild flavour that has been described as oily, and a faint smell reminiscent of radishes. The spore print is white, and the oval spores measure 5.9–6.8 µm long by 4.2–5.1 µm wide.<sup>[1]</sup> *Calocybe indica* grows in grasslands, fields and road verges in Tamil Nadu and Rajasthan, generally on a substrate that is rich in organic material. The mushrooms appear between May and August after spells of rainfall. The fungus is saprophytic, though it has been reported to form ectomycorrhizal relationships with the roots of the coconut tree (*Cocos nucifera*), palmyra palm (*Borassus flabellifer*), tamarind (*Tamarindus indica*) and yellow poinciana (*Peltophorum pterocarpum*).<sup>[2]</sup> *Calocybe indica* is cultivated commercially in southern India and becoming more popular in China, Malaysia, and Singapore; it can be grown in hot humid (60% to 70%) countries with a temperature range of 25 to 35 °C year-round.<sup>[2]</sup> *Calocybe* is a small genus of about 40 species of mushroom,<sup>[1]</sup> including St. George's mushroom, which is edible, and milky mushroom, which is edible and is cultivated in India.<sup>[2]</sup> There are not many species of this genus in Britain. The name is derived from the Ancient Greek terms *kalos* "pretty", and *cubos* "head".<sup>[3]</sup> Around nine species are found in neotropical regions.[1,2,3]

This is the first indigenous mushroom to be commercialized in the country. Mushrooms white in color, gills and stalks white. Mushrooms large with long, thick fibrous stalk. It can be grown on pasteurized or sterilized wheat or paddy straw. Optimum temperature requirement for spawn running 30-35°C. Spawn running period 24-28 days. Casing essential for fructification. Cropping requires an optimum temperature of 35-38°C, humidity of 85-90%, diffused light and ventilation. Mushrooms can be harvested in 2 flushes after which the entire cycle is repeated. Total cultivation cycle of 57-60 days. Biological efficiency potential 50-100%. Shelf life 2-3 days at 25-30°C and 10-15 days at 4°C. It can be marketed as fresh, dry or as mushroom powder. Spent mushroom substrate (SMS) can be used as an excellent organic manure or for vermicomposting. It is an excellent edible mushroom with high fiber.

Important special traits:

- High Temperature Species
- Excellent Shelf Life And No Browning
- High Fiber Content

## DISCUSSION

Milky mushroom (*Calocybe indica*) can be grown on wide range of substrates as in case of oyster mushroom. It can be grown on substrates containing lignin, cellulose and hemicelluloses. Substrate should be fresh and dry. Substrates exposed to rain or harvested premature (green colour) are prone to various weed moulds which may result in failure of the crop. It can be grown on straw of paddy, wheat, ragi, maize/bajra/cotton stalks and leaves, sugarcane bagasse, cotton and jute wastes, dehulled maize cobs, tea/coffee waste etc., However cereal straw (paddy/wheat) easily available in abundance, is being widely used. Straw is chopped in small pieces (2-4cm size) and soaked in fresh water for 8-16 hours. This period can be reduced when pasteurization is to be done by steam. Main purpose of soaking is to saturate the substrate with water. It is easier to soak if straw is filled in gunny bag and dipped in water. The purpose of pasteurization is to kill harmful microbes. This can be achieved in two ways. Water is boiled in wide mouth container and chopped wet straw filled in gunny bag is submersed in hot water for 40 minutes at 80-90°C to achieve pasteurization. This is very popular method particularly with small growers. Wet straw is filled inside insulated room either in perforated shelves or in wooden trays. Steam is released under pressure from a boiler and temperature in side substrate is raised to 65°C and maintained for 5-6 hours. Air inside the room should be circulated to have uniform temperature in the substrate. Substrate is filled in polypropylene bags (35x45cm, holding 2-3 kg wet substrate) and sterilized at 15 lb psi for 1 hour. Once pasteurization/sterilization is over straw is shifted to spawning room for cooling, bag filling and spawning.[4,5,6]

Casing means covering the top surface of bags after spawn run is over with pasteurized casing material in thickness of about 2-3cm. Casing provides physical support, moisture and allows gases to escape from the substrate. Casing material (soil 75% + sand 25%) with pH adjusted to 7.8-7.9 with chalk powder is pasteurized in autoclave at 151b psi for one hour or chemically treated with formaldehyde soln (4%) about a week in advance of casing. Solution should be enough to saturate the soil. It is covered with polythene sheet to avoid escape of chemical and at a interval of 2 days soil is turned so that at the time of casing soil is free from formalin fumes. Bag's top is made uniform by ruffling top surface of the substrate and sprayed with solution of carbendazim (0.1%) + formaldehyde (0.5%). Casing material is spread in uniform layer of 2-3 cm thickness and sprayed with solution of carbendazim and formaldehyde to saturation level. Temperature 30-35°C and R.H. 80-90% are maintained. It takes about 10 days for mycelium to reach on top of casing layer when fresh air is introduced while maintaining temperature and R.H. as above. Light should be provided in long time. The changes thus made in environment, result in the initiation of fruiting bodies with in 3-5 days in the form of needle shape which mature in about a week. Mushrooms 7-8cm diam. are harvested by twisting, cleaned and packed in perforated polythene/polypropylene bags for marketing. Mushrooms can also be wrapped in klin film for longer storage.

Milky mushroom (*Calocybe indica*) can be grown on wide range of substrates as in case of oyster mushroom. It can be grown on substrates containing lignin, cellulose and hemicelluloses. Substrate should be fresh and dry. Substrates exposed to rain or harvested premature (green color) are prone to various weed moulds which may result in failure of the crop. It can be grown on straw of paddy, wheat, ragi, maize/bajra/cotton stalks and leaves, sugarcane bagasse, cotton and jute wastes, dehulled maize cobs, tea/coffee waste etc., However cereal straw (paddy/wheat) easily available in abundance, is being widely used.

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#### Pasteurization

The purpose of pasteurization is to kill harmful microbes. This can be achieved in two ways.

#### Hot water treatment

Water is boiled in wide mouth container and chopped wet straw filled in gunny bag is submersed in hot water for 40 minutes at 80-90C to achieve pasteurization. This is very popular method particularly with small growers.

#### Steam pasteurization

Wet straw is filled inside insulated room either in perforated shelves or in wooden trays. Steam is released under pressure from a boiler and temperature in side substrate is raised to 65C and maintained for 5-6 hours. Air inside the room should be circulated to have uniform temperature in the substrate.

#### Sterilization

Substrate is filled in polypropylene bags (x45cm, holding 2-3 kg wet substrate) and sterilized at 15 lb psi for 1 hour. Once pasteurization/sterilization is over straw is shifted to spawning room for cooling, bag filling and spawning.

#### Spawning and spawn running

Spawning methods are similar to that mentioned in case of oyster mushroom. In case of sterilization of pp bags in autoclave either surface or through spawning should be done. Higher spawn dose 4-5% of wet substrate is used. After spawning bags are shifted to spawn running room and kept in dark where temperature 25-35C and relative humidity above 80% are maintained. It takes about 20 days when substrate is fully colonised and bags are ready for casing. Bags are shifted to cropping room for casing and cropping.[7,8,9]

#### Casing

Casing means covering the top surface of bags after spawn run is over, with pasteurized casing material in thickness of about 2-3cm. Casing provides physical support, moisture and allows gases to escape from the substrate. Casing material (soil 75% + sand 25%) with pH adjusted to 7.8-7.9 with chalk powder is pasteurized in autoclave at 15lb psi for one hour or chemically treated with formaldehyde soln (4%) about a week in advance of casing. Solution should be enough to saturate the soil. It is covered with polythene sheet to avoid escape of chemical and at a interval of 2 days soil is turned so that at the time of casing soil is free from formalin fumes. Bag's top is made uniform by ruffling top surface of the substrate and sprayed with solution of carbendazim (0.1%) + formaldehyde (0.5%). Casing material is spread in uniform layer of 2-3 cm thickness and sprayed with solution of carbendazim and formaldehyde to saturation level. Temperature 30-35C and R.H. 80-90% are maintained.

#### Cropping

It takes about 10 days for mycelium to reach on top of casing layer when fresh air is introduced while maintaining temperature and R.H. as above. Light should be provided in long time. The changes thus made in environment, result in the initiation of fruiting bodies with in 3-5 days in the form of needle shape which mature in about a week. Mushrooms 7-8cm diam. are harvested by twisting, cleaned and packed in perforated polythene / polypropylene bags for marketing. Mushrooms can also be wrapped in kiln film for longer storage.



## RESULTS

*Calocybe indica* belongs to a small genus containing about 40 species of mushrooms (ref. to Wikipedia for *Calocybe*). They have a milky white color with a cap that can grow as large as 15cm in diameter and a stem which may exceed 30cm in length. One mushroom can weigh as much as 2kg – under optimal environmental conditions and using suitable substrate. Cap may be Campanulate -i.e. bell shaped or convex in the initial stages of development and flattens as it grows further and becomes ready to be harvested. Gills are sinuate or smoothly attached and run down the stem. Spore print is white. The size and shape of the cap during cultivation, depends mainly on the light and the oxygen availability as well as the amount of substrate available.

When cooked, mushrooms remain firm with a texture similar to chicken and a mushroom flavor. It is very suitable for Vegetarian Sindhi Biryani – recipe of which shall appear in this blog soon.

This mushroom variety was first identified in the eastern Indian state of West Bengal and can be cultivated on a wide variety of substrates, at a high temperature range (30~38°C). However, no commercial cultivation was made until 1998. Krishnamoorthy 1997 rediscovered the fungus from Tamil Nadu, India and standardized the commercial production techniques for the first time in the world. This edible mushroom has a long shelf life (5~7 days) compared to other commercially available counterparts. A comprehensive and critical review on physiological and nutritional requirements *viz.*, pH, temperature, carbon to nitrogen ratio, best carbon source, best nitrogen source, growth period, growth promoters for mycelia biomass production; substrate preparation; spawn inoculation; different supplementation and casing requirements to increase the yield of mushrooms has been outlined. Innovative and inexpensive methods developed to commercially cultivate milky white mushrooms on different lignocellulosic biomass is also described in this review. The composition profiles of milky white mushroom, its mineral contents and non-enzymatic antioxidants are provided in comparison with button mushroom (*Agaricus bisporus*) and oyster mushroom (*Pleurotus ostreatus*). Antioxidant assay results using methanol extract of milky white mushroom has been provided along with the information about the compounds that are responsible for flavor profile both in fresh and dry mushrooms. Milky white mushroom extracts are known to have anti-hyperglycemic effect and anti-lipid peroxidation effect. The advantage of growing at elevated temperature creates newer avenues to explore milky white mushroom cultivation economically around the world, especially, in humid tropical and sub-tropical zones. Because of its incomparable productivity and shelf life to any other cultivated mushrooms in the world, milky white mushroom could play an important role in satisfying the growing market demands for edible mushrooms in the near future.

Currently, the milky white mushroom farming is done manually. The cultivation process is labor intensive and fairly energy demanding. The crop production process involves six different steps *viz.*, (1) spawn production, (2) substrate pretreatment, (3) mushroom bed preparation, (4) cropping room maintenance during spawn run and mushroom production, (5) harvesting and packaging, and (6) management of spent mushroom substrate. Farmers normally use milled paddy straw as substrate (2~4 cm) The milled straw is soaked in water for 4~5 hr, prior to hot water (80°C) or steaming treatment for 45~60 min. After pre-treatment, the materials are shade dried to get appropriate moisture condition (60~70%) before bed preparation. Polypropylene bags (60 × 30 cm) are normally used as containers for bed production and layer spawning with grain spawn is typically the adopted technique. For spawn run, the bags are kept in clean rooms maintained at 25~30°C and 80~85% humidity for 15~20 days. At this stage, steam-treated casing soil is applied on half-cut beds to a depth of 1.5~2.0 cm. The beds are then transferred to cropping rooms (polythene sheet covered rooms) maintained at 30~35°C, with humidity of higher than 80%. Sufficient natural light should be made available inside the cropping room. Pin heads will appear on the casing surface within a week and the mushrooms attain harvesting maturity in a couple of days. The first flush of mushrooms will normally appear within 24~30 days of bed preparation. Over a period of 40~45 days, mushrooms could be harvested in three to four flushes [10,11,12]

The six major constituents of mushrooms are water, proteins, carbohydrates, dietary fiber, fat, and ash [33]. The moisture content of mushrooms is usually determined by drying at 105°C in a hot air oven overnight to a constant weight. The difference in weight before and after drying is expressed in terms of percentage. The protein content is determined by Kjeldahl method and the lipids are estimated by Twisselman method using extractive solvent like diethyl ether [36]. The lipids in mushrooms include free fatty acids, mono-, di-, and triglycerides, sterols, sterol esters and phospholipids. The sporophore samples are incubated in a muffle furnace at 500°C to estimate the ash content which normally contains potassium and phosphorous. Total carbohydrate content in a given mushroom sample is calculated using the formula, 100 - moisture (%) - protein (%) - crude fat (%) - ash (%) and expressed as g/100 g of fresh or dry sample [37]. According to Crisan and Sands, the energy content in mushrooms is influenced by the composition of

crude protein, fat and carbohydrates whose conversion factors are 2.62, 8.37, and 3.50 kcal/g of the individual components, respectively [38]. These conversion factors are slightly lower than the actual conversion factors used for other food ingredients because they are estimated as crude components.

Sivaprakasam and Doshi et al. [38,39], recorded 20.2% protein from the caps of milky white mushroom (on dry weight basis). Krishnamoorthy et al. [6] reported 32.2% protein (dry weight basis) in a medium sized milky mushroom. Interestingly, among the three mushrooms viz., button, oyster, and milky white mushrooms, the latter has been reported by several authors to contain more protein [6]. Milky white mushroom also has lower moisture content (85.6%) and increased fiber content (61.1% on dry weight basis). Total carbohydrates and crude fat were also found to be more abundant in milky mushrooms (59.9% and 0.67%, respectively) when compared to oyster mushroom varieties (*P. eous* and *P. sajor caju*). These differences could be due to variations in mushroom culture conditions, time of sampling and substrates used. Saranya et al. [40] have reported that the type of substrates and supplements used for mushroom cultivation had greatly influenced the proximate composition (carbohydrate, protein, fat, fiber, ash content, and moisture content) including antioxidants. Based on the availability of soluble sugars. These authors also reported increased levels of calcium, phosphorus and iron in the milky white mushroom. Similarly, total lipids, carbohydrates and ash contents in milky white mushroom were also found to be comparatively higher, ultimately increasing its total calorific value (50.03 kcal/100 g). The dietary fiber (fungal cell wall components mostly, chitin-N-acetyl-glycosamine units) and protein contents of milky white mushrooms are higher than button mushroom, but lower than oyster mushroom. It has been reported that chitosan from *C. indica* sporophores ranged from 2.5% to 2.9% on dry weight basis [41]. In case of *P. florida*, an oyster mushroom species, it ranged from 2.0% to 2.3%. The FTIR spectrum of the fungal chitosan obtained from *C. indica*, *P. florida* and shrimp chitosan exhibited eight major peaks at the ranges of 3,000~3,500/cm, 3,426/cm, 2,885/cm, 1,650/cm, 1,589/cm, 1,400~1,650/cm, 1,326/cm, 1,080/cm and revealed a strong structural similarity among them. The beta-glycans present in dietary fibers of mushrooms are reported to have stimulatory effect on immune system with anti-mutagenic, anticancer and antitumor activities [37]. Mushrooms are good sources of minerals (Ca, K, Mg, Na, and P), trace elements (Cu, Fe, Mn, and Zn) and sometimes, toxic heavy metals (Cd and Pb) as compared to vegetables [43].

we characterized the extracted bioactive compounds from *Calocybe indica* using chromatographic techniques with a modified solvent evaporated extraction method and intended to investigate the polyphenolic profiling. Based on the fragmentation pattern of mass spectra and their direct comparison with mass spectra as well as with the chemical profiles revealed the structure of the obtained compound. (Najda et al., 2021).[16,17,18,19] High-Performance Liquid Chromatography is a precise analytical technique for the separation and identification of biological substances in a mixed solution used for the quantification of individual compounds and accurately determines each compound based on peak area and concentrations (Kuppusamy et al., 2018). Inflammation is the natural and necessary reaction by the body to signals caused by tissue damage or any pathogenic infestation primarily arises as a series of events, beginning with a quick induction phase that results in a pro-inflammatory response, followed by a resolution phase (Najda et al., 2021). As a result, inflammation is a fundamental key to wound healing, however, if this well-coordinated response is disrupted, inflammation can become uncontrolled or chronic, leading to the development and progression of a variety of inflammatory disorders such as cancer, obesity, sepsis, cardiovascular, neurological and autoimmune diseases (Bains et al., 2021). Therefore, the present study was carried out based on the following objectives: i) Formulation and characterization of the *Calocybe indica* powder, ii) evaluation of antimicrobial efficacy of *Calocybe indica*, and iii) determination of anti-inflammatory of *Calocybe indica* extract.[13,14,15]

## CONCLUSION

Recent interest in the utilization of mushroom-based bioactive compounds has increased due to their potential bioactivities and as alternatives in the reduction of high concentrations of chemical utilization. Therefore, we evaluated the physicochemical, functional, antimicrobial, and anti-inflammatory activity of the *Calocybe indica*. The nutritional composition of the mushroom was found to be a good source of proteins (12.48%) and fiber (6.87%). Polysaccharide and protein moiety showed both hydrophilic and hydrophobic domains and the sample showed higher water (3.01 g/g), oil binding (2.45 g/g) emulsifying (68.94), and foaming properties (59.39%). Structural characterization revealed the porous and small crystalline structure of the mushroom powder. Ethanolic extract was quantified for total phenolics and flavonoids and revealed 11.1534 ppm caffeic acid, 0.057 ppm syringic acid, 1.6385 ppm p-coumaric acid, and 0.3495 ppm rutin, respectively. Presence of ethyl tridecanoate, hexadecanoic acid ethyl ester, pentadecanoic acid ethyl ester,

undecanoic acid ethyl ester, N,  $\alpha$ ,  $\alpha'$ -trimethyl diphenethylamine, nicotinonitriles, phosphonic acid decyl-, 1-hexyl-2-nitrocyclohexane, diallyl divinylsilane, 3-phenyl-pyrrolo(2,3- $\beta$ ) pyrazine was confirmed during GC-MS analysis. Furthermore, the mushroom extract showed effective antimicrobial against Gram-positive (23.67 mm) and negative bacteria (20.33 mm) in terms of zone of inhibition. Significantly comparable anti-inflammatory activity was observed for mushroom extract during protein denaturation (43.72-85.69%) and membrane stabilization. In conclusion, the mushroom extract has shown good functional properties and potential bioactivity, therefore, it can be scaled up as an effective food preservative, potential anti-inflammatory, and antimicrobial agent at the industrial level.[20]

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