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Identification and nutritional characterization of a wild *Calocybe indica* strain from Anantapur forest

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ABSTRACT

Knowledge pertaining to the edibility of wild mushroom species is increasing due to immense interest in foraging and traditional foods. Mushrooms are also known as "vegetable meat" and cherished for their culinary delicacies all around the world. A milky mushroom (Calocybe indica) strain was collected and isolated from wooden logs near agriculture research station, Reddipalli village, Bukkarayasamudram mandal, Anantapur, Andhra Pradesh, India. The specimen was tissue cultured on potato dextrose agar media to obtain pure culture. The Identity of the specimen was confirmed by ITS sequencing. Microscopic observations revealed the presence of clamp connection in mycelium. Nutritional evaluation was conducted with respect to carbohydrate, proteins, ash, fibres, fat and total phenol by various assays. The results revealed high carbohydrate (45.25g/100g) and protein (25.67g/100g) along with an energy value of 314.91 Kcal.

Keywords: Mushroom, *Calocybe indica*, ITS sequencing, phylogeny, nutritional value

Mushrooms are generally the fruiting bodies of filamentous saprophytic macroscopic fungi that grow over the ground. Their valuable effects on human health and nutrition value go back to early China, Egypt, Greek and Roman civilization (Das *et al.,* 2021). There are 1.5 million fungi assessed, around 2000 species are reported to be edible and 25 species are accepted for commercially cultivation (Niego *et al.,* 2021). *Agaricus, Lentinula* and *Pleurotus* genera of mushroom are most widely cultivated and commercialized around the world (Assemie *et al.,* 2022; Valverde *et al.,* 2015).

Interestingly, mushrooms are regarded as domain fungi and some of the unique characters of these domain fungi are eukaryotic, haploid, nucleated, nonvascular, cryptogamic, heterotrophic, gametophytic, achlorophyllous organisms with chitin as its cell wall (Boda *et al.,* 2012). The classification of mushrooms was done using standard procedure in taxonomic identification that follows Phylum Basidiomycota, Division Eumycota, Subdivision Basidiomycotina and Class Hymenomycetes (Ukwuru *et al.,* 2018).

Mushroom fruit bodies are consumed as food for its unique taste, aroma and its nutritional values (Guillamon *et al.,* 2010). The growth of mycelium and fruiting body can be affected by various factors like light and dark events, light intensity, composition of

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air, rate of air flow, temperature, humidity, composition of substrate and also species (Shoji and Kitamoto, 1997).

Due to their important metabolic content, mushrooms are also used in cosmetic, pharmaceutical, and medicinal applications (Selem *et al.,* 2021). Mushrooms are also considered as a suitable substitute to muscle protein due to their high digestibility (Kalac *et al.,* 2012). With advances in modern agriculture, we are now self-reliant for food but we are still struggling as far as nutritional composition of food is concerned. The exploration of edible mushroom for its nutritional values is a viable option and is still an underdeveloped concept in our country against China, where people have grown and consumed them from 600 AD (Yu *et al.,* 2020). Consumption of mushroom world-wide is on rise as more people are recognising its value as healthy food (Sifat *et al.,* 2020). Mushroom being an excellent source of vitamin D & vitamin B12 (Shah*,* 2021), low in calories, gluten free, fat free, cholesterol free and very low sodium content. It also contains minerals like copper, iron, potassium, manganese, and zinc (Sharma *et al.,* 2017). Diversification in any system impart sustainability including agriculture in general and mushroom cultivation in particular. India is a country with diverse climate and rich germplasm diversity but we have still not utilized our own germplasm diversity fully.

This study reports a wild strain of milky mushroom (*Calocybe indica*) and its nutritional values to add the strain to the mushroom basket of our country.

MATERIALS AND METHODS

Collection of germplasm

Mushroom strain was collected and isolated from wooden logs near agriculture research station, Reddipalli village, Anantapur, Andhrapradesh, India.

Microscopic examination

Collected specimen was worked out for their microscopic examination by teasing specimen and using Lactophenol blue stain and observed under microscope under oil immersion.

Raising pure culture

Pure culture was raised on PDA slants and Petri dishes under aseptic conditions and incubated at 24+1 ºC for future use.

Isolation of genomic DNA

The genomic DNA was isolated from fungal isolate following the method of Moller *et al*., (1992). The fungal culture was grown in 100 ml PDB for 5 days. The harvested mycelial mat was ground finely in pestle and mortar using liquid nitrogen. About 50 mg of the powdered mycelium was transferred into a microtube contained 500 µl of pre-warm TES (100 mM Tris, pH 8.0, 10 mM EDTA, 2% SDS). To which, 50 µg proteinase K was added and incubated for 1 h at 60°C with occasional gentle mixing. To the above mixture, 140 µl of 5M NaCl was added to adjust the salt concentration to 1.4 M. Now 65 µl of 10% CTAB (Cetyl Trimethyl ammonium bromide) was added and incubated for 10 min at 65°C. To the above mixture, 700 µl of Chloroform and isoamyl alcohol (24:1) was added, mixed gently, incubated for 30 min at room temperature and centrifuged at 10000 rpm for 10 min at 4°C. The supernatant was transferred to a 1.5 ml tube, to which 225 µl of 5 M ammonium acetate was added, mixed gently, incubated on ice for 30 min and centrifuged at 10,000 rpm for 15 min at 4°C. The supernatant was transferred to a fresh tube, 510 µl of isopropanol was added to precipitate the DNA and centrifuged immediately for 10 min at 10,000 rpm. The supernatant was removed, the pellet was washed

twice with cold 70% ethanol, air-dried and suspended in 50 µl TE buffer.

Amplification of Internal Transcribed Spacer region

In this study, ITS1: 52 - TCCGTAGGTGAACCTGCGG-32 was used as a forward primer and ITS4: 52 - TCCTCCGCTTATTGATATGC-32 was used as reverse primer (White *et al.*, 1990). Polymerase chain reaction was performed in a thermocycler to produce multi copies of a specified DNA. Amplification was carried out with an initial denaturation at 95°C for 5 min followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min, extension at 72°C for 2 min and final extension at 72°C for 10 min using a thermocycler (iCycler; Bio-Rad Laboratories, CA). PCR products were analyzed on 1% agarose gel to visualize amplicons. The amplified product was sequenced using ABI PRISM 3730 Genetic Analyzer (Applied Biosystems).

Sequence analysis

The sequences of these ITS regions were compared against the sequences available from GenBank using the BLASTN program (Zhang *et al.,* 2000) and were aligned using CLUSTAL W software (Thompson *et al.,* 1994). Distances were calculated according to Kimura's two-parameter correction (Kimura, 1980). Phylogenetic trees were constructed using the UPGMA method (Sneath and Sokal, 1973). Bootstrap analysis was done based on 1000 replications (Felsenstein, 1985). The MEGAX package (Kumar *et al.,* 2018) was used for all analysis.

Temperature optimization

Studies were carried out to find out the optimum temperature for the growth of this mushroom on PDA medium at different temperatures i.e. 21, 25 and 30 ^{o}C. The study was carried out in triplicate.

Preparation of spawn

Pure culture of the mushroom was used for spawn preparation on Sorghum grains. The grains were soaked, boiled and mixed with 1% Gypsum and 1% chalk powder. The grains were filled in bottle up to 2/3rd and plugged with nonabsorbent cotton, the plugs are covered with aluminum foil and sterilized for 2 hours at 22 lb psi in an autoclave. Bottles were left to cool and inoculated with a piece of mycelium grown in Petri dish and incubated at 25 °C (Sharma *et al.*, 2005). The mushroom was cultivated following the standard protocol. The fruit bodies were used for further analysis.

Nutritional evaluation

800 gm mushroom was weighed and dried at 50 ± 5 °C till constant weight was achieved. This process was carried out for 3days continuously with an average of 9 hrs per day (Piskov *et al.*, 2020). The dried sample was subjected to crushing followed by passing it to a sieve with the mesh size of 0.9 mm. Finally, the dry powder was collected and stored in a cool and dry place for further analysis. (Yu *et al.,* 2020)

Determination of moisture content

The determination of moisture content was carried out by the traditional gravimetric method. 20 g of fresh mushroom was taken and added to moisture can. Both the can and the sample were oven dried at 105°C for 5 to 6 hours. It was then cooled in a desiccator and reweighed for the difference. The observed weight was recorded while the sample was returned to the oven for further drying for another half to one hour. The drying, cooling and reweighing was done repeatedly until a constant weight was obtained. (Alam *et al.*, 2008).

$$\% \text{ Moisture} = \frac{\text{initial weight - final weight}}{\text{weight of the sample}} \times 100$$

Determination of total ash

Firstly, Porcelain crucible was subjected to heat at temperature 600 °C for 30 min in a muffle furnace. It was cooled to room temperature using desiccator. Then the crucible was weighed. The powdered sample of about 5g was weighed and transferred to tared crucible and was placed in muffle furnace and the temperature was maintained to 600°C for 16 hours, the ash was reweighed along with the crucible (Yang *et al.*, 2016).

$$\% \text{ Ash} = \{\text{Weight (sample +crucible) -Weight(crucible) / weight (sample)}\} \times 100$$

Determination of Phenol content

Dried and powdered sample (5.0 g) was extracted with 25 ml ethanol. The extraction was carried out for 7 days accompanied by occasional shaking and stirring. The extracts were filtered through cotton material and later through Whatman No. 1 filter paper. The residues were extracted again with 10 ml of ethanol. The combined extracts were evaporated to dryness under reduced pressure by a rotary vacuum evaporator. The dried extracts were stored at 4°C until used for analysis.

The extract was re-dissolved in 10 ml ethanol and 0.3 ml of the extract was mixed with 1.2 ml of the Folin-Ciocalteau reagent solution (10%, v/v) and 1.5 ml of sodium carbonate solution (7.5% w/v). The absorbance was measured at 765 nm after incubation of the reaction mixture for 1 hour. A calibration curve was constructed with varying concentrations (60-150 µg/ml) of gallic acid as standard. The results were expressed as mg of gallic acid equivalents (GAE) per gram extract (Sánchez *et al.*, 2013).

Determination of total protein content

Protein content was determined by Kieldahl method, adapted by AOAC. accurately weighed 1 gram of sample was hydrolyzed with 15ml conc. sulfuric acid with two metal (selenium and copper) catalyst in a digester at 420 for about 2 hours and cooled. The digested sample was distilled with 45% NaOH and distillate was collected in 4% boric acid solution with a drop of mixed indicator solution. Further the distillate was titrated with 0.1N HCl and the total nitrogen was calculated using the formulae

$$\text{Percentage nitrogen} = \frac{\text{(X-Y) x N x 0.014 x V1}}{\text{V2 x W}} \times 100$$

V1 = Volume of aliquot (100 ml)
V2 = Aliquot taken for distillation (10 ml)
N = Normality of HCl (0.1 N)
X = Volume of HCl used for titration of sample
Y = Volume of HCl used for titration of blank

The total nitrogen was then multiplied with traditional conversion content 6.25 and species-specific conversion factor to calculate the total protein content present in the sample. The result was expressed in g/100gm (Maehre *et al.*, 2014).

Determination of crude fiber

The total dietary fiber was determined by Enzymatic gravimetric method. The sample taken was 20g and transferred to 400 ml beakers. 40ml MES-TRIS blend buffer solution was added to each beaker and was stirred until the sample was completely dispersed. After the completion of all enzymatic digestion by heat-stable alpha amylase, protease and amino glucosidase. About 280 ml of 90% ethyl alcohol was added and was allowed to form precipitate. Test portion (for ash) was incinerated at 525°C for 5 hours.

Total dietary fiber = weight (residue)- weight (ash protein)

Determination of total lipid

About 5grams of the dried mushroom sample was added to cellulose thimble followed by placing the thimble in the Soxhlet chamber, which was prior fitted to a tared distillation flask that contained 80 ml of hexane along with a boiling glass regulator. After lipid extraction for 16 h, the solvent was vaporized by a rotary-evaporator and the remaining traces were removed by heating it at 80 ºC overnight. The flask was cooled and weighed. This heating-reweighing step was repeated until the approximate difference between two consecutive weighing was less than 10 mg. The result was expressed in g/100g (AOAC 20TH Edn.2016, 920.39).

Total carbohydrate estimation

The content of the available carbohydrate was determined by calculation the formulae adopted is written below (Alam *et al*., 2008).

Carbohydrate (g/100 g sample) = {100 - (moisture + fat + protein + ash + crude fiber)}/100

Determination of energy available

Energy available was determined by multiplying number of grams of protein, carbohydrates and fat by 4, 4, and 9, respectively.

Energy available (Kcal/100g) = (Number of grams of carbohydrates × 4) + (Number of grams of protein × 4) + (Number of grams of fat × 9).

RESULTS AND DISCUSSION

The sporocarps of the wild specimen were collected in paper bags and the field notes like date, location, GPS coordinates, weather conditions, habitat, etc. were recorded. Fresh samples were used to document morphological traits, and the samples were subsequently taken to the lab for taxonomic attribution and culture isolation. Macroscopic observation revealed the presence of cap, stalk, gills but absence of annulus and scales (Table 1).

The cap or the pilus was broadly convex, white, as the apex of the pileus was broadly flattened. Shape of the pileus was more or less round (orbicular). The edge of the cap was curled downwards and inwards toward itself (involute). Habitat was tropical forest of Anantpur (Andhra Pradesh). The fruiting bodies grow close together in group and more importantly do not share a common base as documented in the past review literature (Poorniammal *et al*., 2018). The specimen had white, sinuate, crowded and eroded gills. The gills were numerous and closely arranged. They were sinuate as the gills are notched near the stipe. The margin of the gills is torn in an irregular pattern and is called eroded.

Table 1. Summary of macroscopic features of edible mushroom

S. No	Macroscopic characteristics	Description
1	Cap/Pileus	Broadly convex, white, entire, Orbicular, Involute, Dull.
2	Stalk/ Stipe	Centrally attached, white, clavate, reticulate, fleshy, solid and basal tomentum.
3	Gills	White, sinuate, crowded gills, eroded.
4	Annulus/ Ring	absent
5	Scale	Absent
7	Attachment and texture of fruiting body	Stipitate, Smooth
8	Habit Habitat	GregariousTropical

The average diameter of each structure is a) Pileus (diameter) 6.3cm. b) Stipe (length) - 13.5 cm. c) Total length-15 cm. d) Width of stipe (bulged part- diameter) -5.5cm.

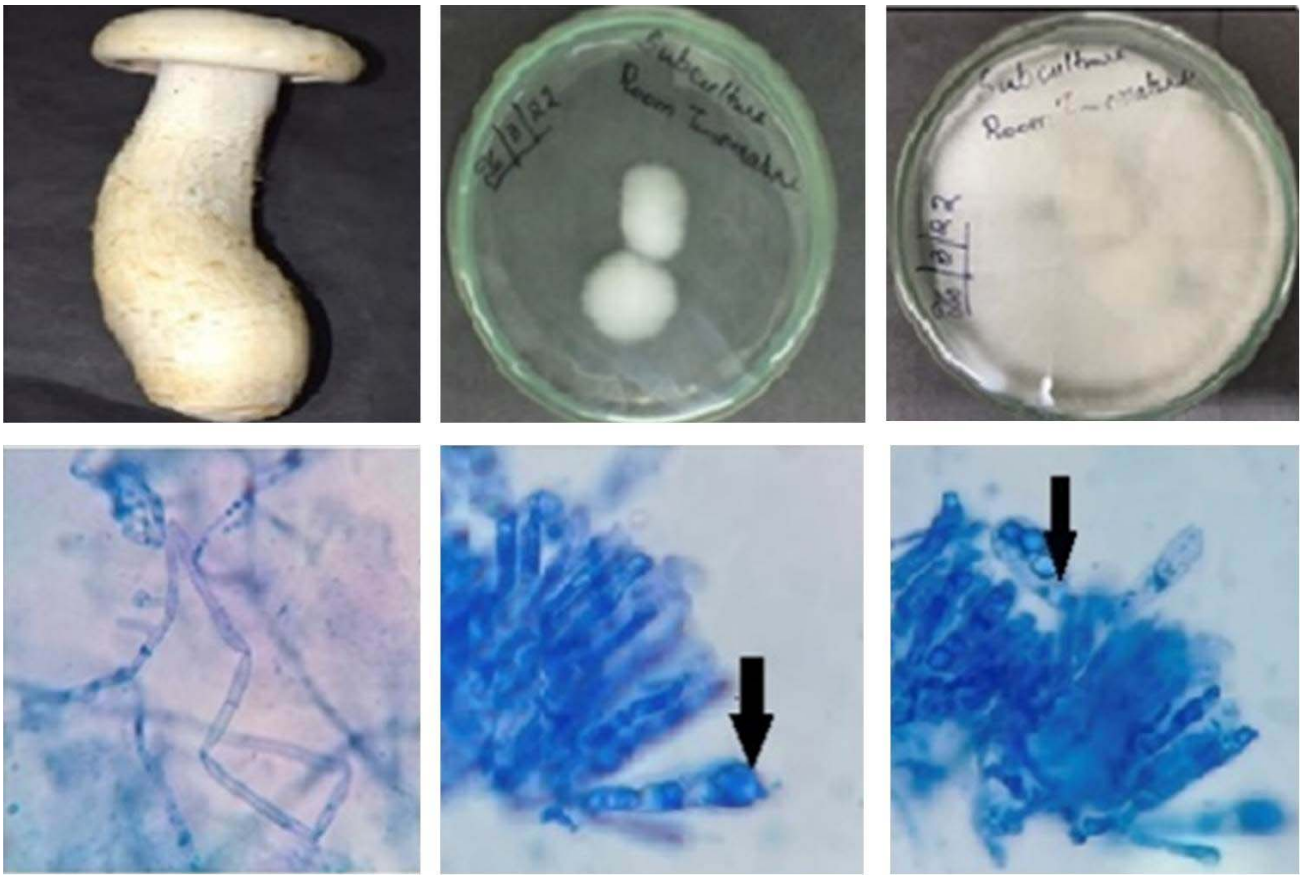


Fig. 1. Colony characteristics and mycelial characters of the collected specimen

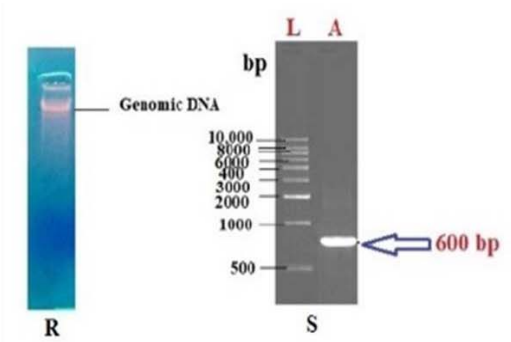
The specimen was cultured on PDA. The mycelium covered the Petri dish (90mm) in 15 days' time. Cotton blue staining showed septa, multinucleate and branched mycelium (Fig 1). Hyphae showed clamp connection, which occurs during karyogamy in basidiomycetes. The colony characteristics was noted and are given in Table 2.

Table 2. Summary of growth characteristics and colony morphology

	Growth characteristics	
1.	Temperature	25°C - 30°C
2.	Humidity	70-80%
3.	Growth medium	Potato dextrose agar
4.	**Colony Morphology**	
	Shape	Filamentous
	Margin	Filiform
	Elevation	Raised
	Colour	White
	Surface texture (top view)	Cottony
	Colony (bottom view)	Flat, light-yellow spot.

Culture conditions were optimized by growing the mycelium at three different temperatures 21, 25, 30°C and light and dark conditions. The results showed its best growth at 30°C in dark conditions. Hyphal organization of test fungi was septate, branched hyphae and multinucleated.

The identification of the specimen was confirmed using ITS 5.8S rDNA sequencing (Fig. 2). The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal phylogenetic tree showed a sum of branch length = 0.39680377. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown in the Fig. 3



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TCGGTAGGTGAACATGCGGAAGGATCATTAGTGAATTGAACTTGGTCAAGTTGTTGCTGGCTCTCTCAGAGCATGTGC
ACACTTGGCTCTTGTTTCGTAAACCCACTCGTGCACCTTAAGTAGACTTTGCAAGAGTTTGAGTCGAGAGTGATCTTG
GCCCTTACACATCCGAGTCTATGTGTTTTTCATATCATTTTACTCTGTGTATAAGAATGTTTTACTAAGGCATTATTAA
TTCCTTTAAATCATATACAACTTTCAACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATA
AGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTCTGAACGCACCTTGCGCTCCTTGGTATTCCGAGGAGCA
TGCCTGTTTGAGTGTCTTGAAATTCTCAACCTTTGTTATTTTTTTTGTTCAAAGAGTCTGGAAGTGGAGGTTGCTGGCT
TTTGATGAAAGAGTCGGCTCCTCTGAAATACATTAGTGGGACCCATCGTTGATTAGCTCCCTGGTGTGATAGTTATCT
ACGCCGTGGCTCATCACGATATTGTGTGGTTCAGCTTTCTAACGAGACAACAACAAACTGTCTTTGATTATTTTGACC
TCAAATCAGGTAGGACTACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAGAGGGGAGAGAATGAATTGAACTTG
GTCAAGTTGTT-

Fig. 2. ITS rDNA amplification sequencing of the specimen

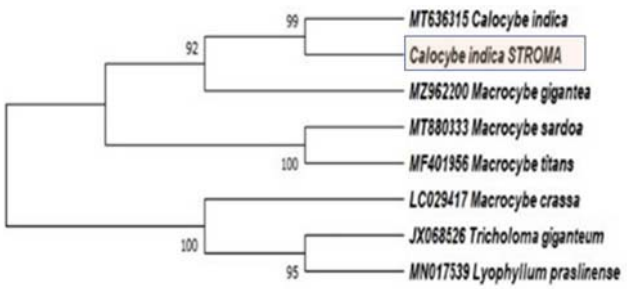

Fig. 3. Phylogenetic tree of the specimen collected with closely associated sequences

(Felsenstein 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and shown as number of base substitutions per site. This analysis involved 13 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion). There were 714 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.,* 2018).

Fig. 4. Different phases of Cultivation of Milky mushroom

During this study, Sorghum seeds were used as substrate for spawn preparation. It took a period of 25- 30 days to completely cover the grains with mycelium. Later, mycelium-coated jowar seeds were used for spawning with paddy straw as a substrate, rice bran and wheat bran were also added which increases the available nutrition. Casing soil (Sand+Soil 1:1) was applied on substrate after complete growth of mycelium in spawned bags. Casing is required because it aids moisture and helps in fructification (Fig 4).

Mushroom fruit bodies were subjected to nutritional analysis and were found to be rich in protein and carbohydrates (Table 3). The mushroom was low in fat like all other mushrooms. It also had 3.547 GAE g equivalent of phenolic content, which shows its high anti-oxidant properties. Dietary fiber content was also found to be 11.69g, which helps decrease cholesterol and helps to reduce diabetics as reported in the literature (Lee *et al*., 2020).

Table 3. Nutritional profile of the mushroom

S. No	Parameters	Per100g sample
1.	Moisture	88.7±0.2 %
2.	Protein content	25.67g
3.	Fat content	3.47g
4.	Crude fibre	11.67g
5.	Ash content	9.78g
6.	Carbohydrate	45.25g
7.	Phenol content	3.547 g GAE
8.	Energy	314.91 Kcal

CONCLUSION

In this study, a strain of *Calocybe indica* was identified on its morphological and molecular characteristics. The strain was also characterized for its nutritional and growth characteristics. The strain can be used for commercial cultivation after repeated production trials. This will add to the mushroom portfolio of country.

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