

## Isolation and Molecular Characterization of Thermophilic Coprophilous Fungus *Malbranchea cinnamomea* GSMBKU from Goat Dung

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### Abstract

Thermophilic fungi, found in the varied habitats are valuable sources for potential enzymes and other biochemical metabolites throwing challenges to the mycologists. A study of such fungi will not only enables the biochemists, microbiologists, and the biotechnologists to understand the basic molecular process but also search for novel genes, gene products and reactions. In this study, *Malbranchea cinnamomea* was isolated from goat dung and characterized based on its morphological and physiological characters, and later confirmed molecularly.

**Keywords:** Thermophilic coprophilous fungus, Habitats, Temperature, characterization, identification.

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## INTRODUCTION

Thermophilic fungi grow in a temperature range of 20°C to 60°C<sup>1</sup>. They are eukaryotic organisms and can grow at high temperatures which makes them easier to study<sup>2,3</sup>. They are a valuable source for various enzymes with commercial and scientific interest<sup>2</sup>. Some of the important industrial enzymes isolated from these fungi include L-Asparaginase, cellulases, proteases, amylases, lipases etc. which can be produced on a large scale commercially<sup>1</sup>. These fungi are also a source of proteins which upon crystallization can help us understand the catalytic mechanism of their intrinsic thermo stability. Another striking feature of these thermostable organisms includes the ability to degrade polysaccharide constituents of biomass<sup>1</sup>. It is estimated that geothermal habitats are the rich source of thermophiles, especially these areas which are abundant with concentrated sulfur, like acidic soils and acidic thermal waters having a variable pH from neutral to alkaline pH<sup>4</sup>. In certain other cases these habitats are characterized by presence of certain gases primarily a composition of nitrogen, carbon dioxide, hydrogen sulfide and hydrogen gas<sup>4</sup>. Life on earth is heterogeneous as is said by Darwin in his theory "survival is the fittest" every flora and fauna adapt to survive in varied conditions found on the earth. These fungi have been reported to be found in natural habitats like soils, air, compost, nesting material, herbivore dung, hot spring, and some of the man-made habitats including pile of wood chips, municipal refugee, peat, pile of hay etc.<sup>5</sup> Thermophilic fungi can be grown by enrichment media by providing ambient temperature specific to taxa<sup>1</sup>. Addition of carbon from different sources shows improved yield<sup>6</sup>.

The non cellulolytic species utilize xylan, mannose and glucose as the carbon source, in certain other cases a combination of sugars like glucose, sucrose and other sugars in various combinations can also be used pertaining of species<sup>6</sup>.

Identification always remain a heavy task if only the morphology is used, which enable us to know the genus only. Hence, the ribosomal RNA genes are most commonly used in the identification which acts as an official DNA bar coding marker for species level identification<sup>7</sup>. A Survey on the natural products secreted by the

fungi was conducted by the Journal of Natural Products says that approximately 31% of fungal identification is solely done by morphology, 28% based on the secondary metabolites secreted, 27% by molecular data like ITS and 14% by combining morphological and molecular data like rRNA and protein coding genes<sup>8</sup>. However accurate identification is required to study further biochemical properties and applications. In the present study thermophilic fungi were isolated from goat dung and characterized.

## MATERIALS AND METHODS

### Chemicals and Reagents

Whatman's filter paper No. 42, Yeast extract, starch, MgSO<sub>4</sub>, K<sub>2</sub>HPO<sub>4</sub>, sucrose, glucose, Agar-agar and other chemicals obtained from the Himedia labs, Mumbai, India.

### Isolation of thermophilic fungi

#### Media and culture condition

The basal medium Yeast extract starch (YES) agar medium was employed for assessing growth

Starch	-15.0g
Yeast extract	- 5.0 g
MgSO <sub>4</sub>	- 0.5 g
KH <sub>2</sub> PO <sub>4</sub>	- 1.0 g
Agar-Agar	-20.0 g
Distilled water	-1000 ml.

Different source of Herbivore (Cow, Buffalo, Deer, Sheep, Goat, Horse, Elephant etc) dung were collected from Warangal district of Telangana, India in sterilized polyethylene bags and were brought to the laboratory and analyzed for the presence of thermophilic fungi followed by paired plate, serial dilution method as described by Cooney and Emerson 1964<sup>9</sup> at 45°C temperature. The fungal colonies developed on Petri plates were isolated and transferred to YESA (yeast extract starch agar) slants, allowed to grow and stored at 4°C for further use.

### Morphological identification and growth optimization

The isolated fungal colonies were then studied to reveal the Microscopic features of strains such as hyphae, conidiophores, branching conidia, pigmentation etc. were determined using a Olympus make microscope (X40) followed by standard manuals of Cooney and Emerson, 1964<sup>9</sup> and Mouchacca, 1997<sup>10</sup>. Thermophilic fungi are

well recognized group by way of temperature relationship and taxonomic characterization<sup>11</sup>. Thermophilic fungi grow at elevated temperatures (50°C or above) are often termed as thermophiles<sup>2,9</sup>.

After incubating the fungus in broth media containing the 50 ml of yeast extract starch without Agar in 100 ml of conical flask for different incubation periods, under the suitable condition at respective temperature, pH and by using different synthetic media included Yeast extract starch (YES), glucose yeast extract (GYE) and czapeck dox modified (CDM). The optimum growth time required for fungus was investigated at various incubation periods (0 day, 3<sup>rd</sup> day, 6<sup>th</sup> day, 9<sup>th</sup> day, 12<sup>th</sup> day & 15<sup>th</sup> day). At the end of incubation period the growth of the fungus from day 0 to day 15 was observed by measuring the dry weight of the biomass to understand the growth of the organism, at each interval the culture filtrate was filtered with the help of Whatmann filter paper NO. 42 (measured (w<sub>1</sub>)) placed on conical funnel. After culture filtration the obtained biomass along with the filter paper was folded and kept for drying in hot air oven (MH Enterprises) and then measured.

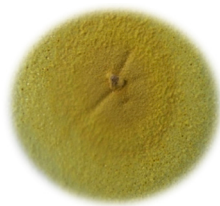
#### Molecular identification

Molecular identification by gene sequencing is important as we can identify the ITS (Internal Transcribed spacer) of fungi that plays a major role in sub generic phylogenetic classification as the locus is specific even within the species<sup>12</sup>. The total genomic DNA isolated from the mycelial mats using Master pure TM Yeast DNA purification kit as described by Jin *et al.*, 2004<sup>13</sup>. D2 region of large subunit of 28S rDNA gene fragment was amplified as described in earlier work<sup>7</sup>. Quiagen quick PCR purification kit was used for purification and sequencing. The sequences were then subjected to BLAST<sup>16</sup> to identify the nearest taxa and aligned with sequences, belonging to the

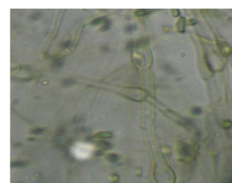
nearest taxa using CLUSTALW<sup>12</sup>. The evolutionary history was inferred using the neighbor –joining method. Phylogenetic tree was constructed using the Kimura 2-parameters using MEGA4 software<sup>14</sup>. Using ITS and rRNA genes to study the taxonomic classification of fungi began 2 decades ago. The fungal DNA sequences for the larger subunit (28S) and the smaller subunits (18S) and the ITS (Internal Transcribed Sequences) accompany the fungal identification. ITS is the most useful and required portion of the rRNA cistron<sup>8</sup>. Protein coding genes are specific to fungal groups and by constructing the phylogenetic trees using the DNA sequences will help in fungal identification<sup>15</sup>. These genes will give an insight into homology, convergence as it is believed that these genes may be species specific<sup>8</sup>. Using PCR (Thermo Scientific make) based techniques the detection of intra and inter specific genetic variations can be done that helps in the identification<sup>16</sup>. These techniques help in evaluating the polymorphisms in the species even with the little differences<sup>7</sup>. These are the molecular markers that are available for the rapid screening of genetic diversity, these markers allow for high resolution of DNA finger printing<sup>17</sup>. Neighbour Joining method and MEGA4 software is faster in term of computing the distances<sup>14,15,18</sup>, estimating genetic distances and diversities, inferring ancestral sequences, computing time trees, and testing selection.

#### RESULTS AND DISCUSSION

Out of all the samples of various herbivore dung collected, goat dung was selected as a personal choice, through in all the samples thermophilic fungi was observed. An only fungus from goat dung was observed for colony morphology and further molecular identification in the present study.



a. Colony

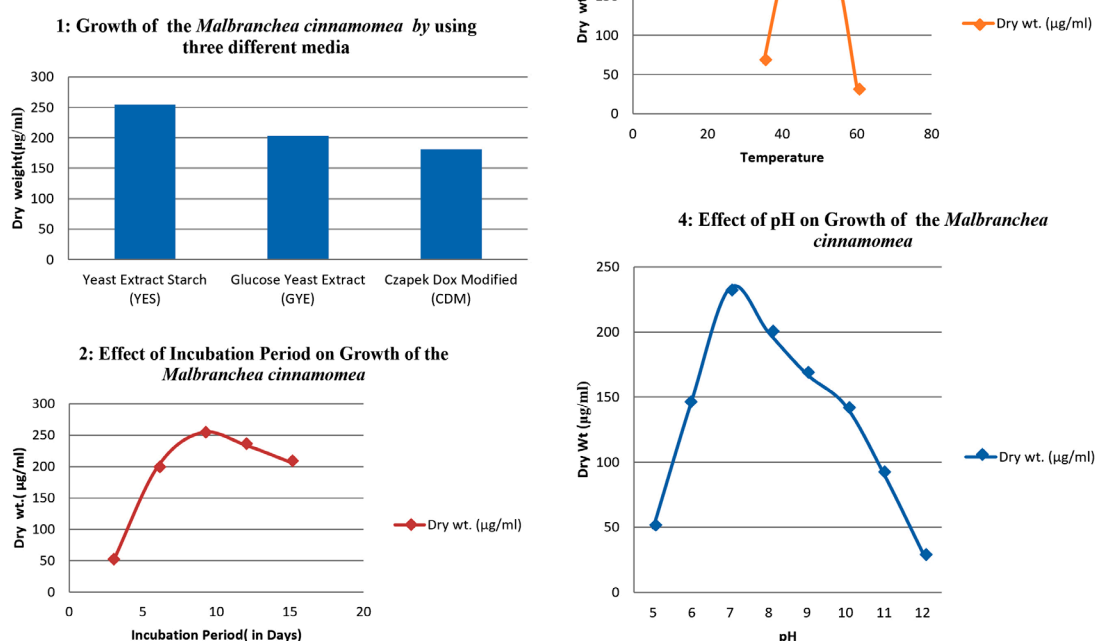


b. Morphology of fungi under compound microscope

**Fig. 1.** *M. cinnamomea*:

*Malbranchea cinnamomea* isolated from goat dung on Yeast extract starch media and the colony observed pigmented whitish-yellow. Further identification was done by observing colony features under compound microscope with the help of standard manuals. Colonies on YESA at 45°C were sulfur yellow robust, dense, thick, smooth with a few outward radiating folds, velvety with coarse, creamy yellow tufts of hyphae. The medium turned black by the diffusion of pigment.

Vegetative hyphae hyaline later became yellowish-brown with racket hyphae, swelling near the septum to a diameter of 9 µm or more. Arthroconidia were observed on curved, lateral



AAACGAAAGGCCCTCACGAAAAAGCACTCAAGCGCGCCCCCGCGGACA  
 CCCTTGTCTACCGAACCACGTTGCCTCGGCGGCCCTTCCCCCGGTGATC  
 GCCCCGGGGGACCGCCGGGGGCGCTCGCCGGCGCCCTGCTGCGCCGACGG  
 CCCCCGGGCCGCGCCCGCCGAGGAGTTCGACCGAACCTCTGGCGATCCG  
 TGAGCGAGTCTGAGTCGGCTGGACAATCGTCAAACTTTCAGCAGCGGAT  
 CTCTTGGTTCCCGCATCGATGAAGAACGCAACGAAATGCGATAAGTAATG  
 TGAATTGCAGAATTCCGTGAATCATCGAATCTTTGAACGCACCTGGCGCC  
 CTCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCAACCCTCAA  
 GCGCGGCTTGTGTGATGGGCGCGCTCCCCCGAACTGGGCGGGACGGG  
 CCCGAAAGGCAGTGGCGGCGTCGCGGTCATCTCGATCGCCCGAGTGCAT  
 GGGGCTCAGTCACGCACGACGGCGAGGACCGGCGCCGGCCGAACCCCGCG

**Fig. 2.** Nucleotide sequences of *Malbranchea cinnamomea*

branches arising from broader vegetative hyphae, 3-6  $\mu$ m in diameter (Fig. 1. a & b).

To study the optimum growth conditions of the organism, three different compositions of synthetic Media including Yeast extract starch (YES), glucose yeast extract (GYE) and czapeck Dox modified (CDM) were used and the results are shown in the graph 1. Their period of growth is also studied at different intervals and represented in the graph 2. The factors affecting the growth of the organism were also studied and the results were tabulated specially the effect of pH and the optimum temperature was 45°C (graph 3 & 4). The lowest growth of the fungus was detected at pH 5.0 and temperature 35°C.

When temperature is concerned, the organism shown a growth at temperatures ranging from 35 to 60°C and the growth was ceased above 60°C. In case of pH the growth was optimum at neutral followed by alkaline conditions. The organism was found to be stable upto pH 12.

Identification of *M. cinnamomea* taxonomic status has employed molecular methods involving PCR to resolve genetic variation. A single discrete PCR amplicon band of 711bp was observed when resolved on agarose gel. Consensus sequences of 632bp of D2 region of LSU gene generated sequence gave the detailed data of the sequence obtained primers are presented in chromatograms data (Fig. 2). The obtained

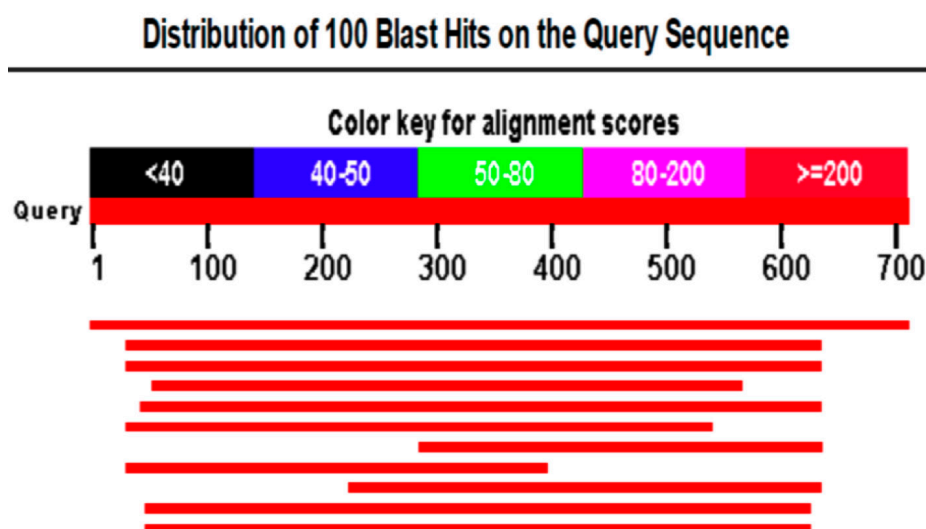


Fig. 3. BLAST analysis of the sequence

Table 1. Distance matrix method

GSMBKU_		0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
KT279416.1											
KJ834332.1	0.68		0.00	0.05	0.06	0.00	0.05	0.05	0.05	0.05	0.05
KJ834368.1	0.68	0.00		0.05	0.06	0.00	0.05	0.05	0.05	0.05	0.05
JF412018.2	0.68	0.68	0.68		0.05	0.05	0.05	0.06	0.05	0.05	0.05
KJ563258.1	0.74	0.69	0.69	0.69		0.06	0.05	0.05	0.06	0.05	0.05
KJ834339.1	0.68	0.00	0.00	0.68	0.69		0.05	0.05	0.05	0.05	0.05
JF922020.1	0.76	0.74	0.74	0.74	0.73	0.74		0.05	0.05	0.05	0.06
EF550978.1	0.69	0.75	0.75	0.56	0.75	0.75	0.78		0.05	0.05	0.05
KP828175.1	0.68	0.78	0.78	0.75	0.63	0.78	0.74	0.70		0.06	0.05
KP278210.1	0.78	0.80	0.80	0.76	0.79	0.80	0.70	0.69	0.71		0.05
KP278209.1	0.70	0.75	0.75	0.70	0.69	0.75	0.74	0.71	0.69	0.68	

DNA sequences data subjected to blast in NCBI and its relatively with ten closest homologs are presented in and alignment view (Fig. 3) the best 10 BLAST hits were taken for phylogentic analysis based on maximum query coverage and maximum identity, one of the sequence from 10 best hit is *Malbranchea cinnamomea*. Based on nucleotide

homology, history of evolutionary distance (table 1) and phylogenetic analysis (Fig. 4) the fungus was confirmed as *M. cinnamomea* and the accession number given by NCBI Gene Bank data base for *Malbranchea cinnamomea* isolate GSMBKU as KT279416.1.

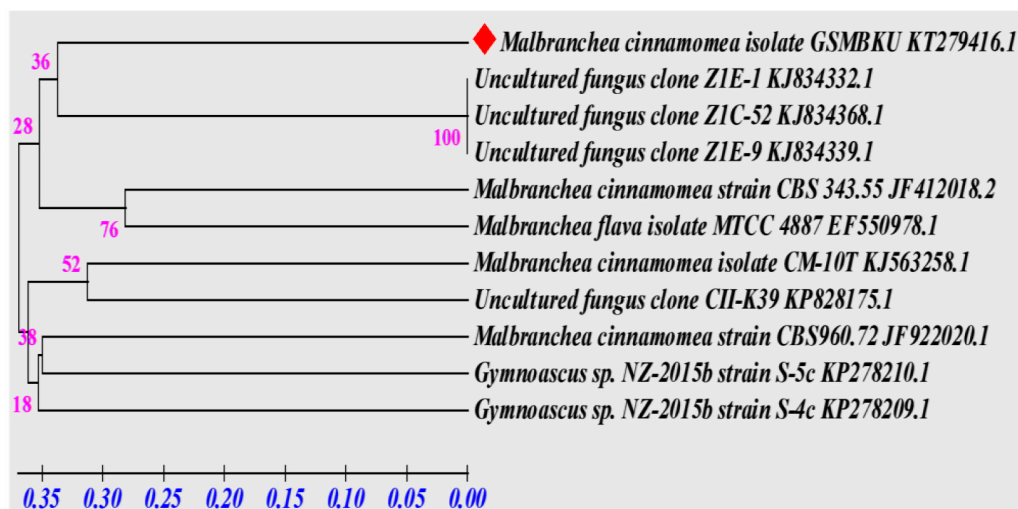


Fig. 4. Phylogenetic tree of *Malbranchea cinnamomea*

Accession	Description	Max score	Total score	Query Coverage	E Value	Max. Identity
KT279416.1	Malbrancheacinnamomea isolate GSMBKU5.8S ribosomal RNA	1314	1314	100%	0.0	100%
KJ834332.1	Uncultured fungus clone Z1E-1 18S ribosomal RNA	1018	1018	84%	0.0	97%
KJ834368.1	Uncultured fungus clone Z1C-52 18S ribosomal RNA	1005	1005	84%	0.0	97%
JF412018.2	Malbrancheacinnamomea strain CBS 343.55 18S ribosomal RNA	876	876	72%	0.0	98%
KJ563258.1	Malbrancheacinnamomea isolate CM-10T 5.8S ribosomal RNA	874	874	82%	0.0	94%
KJ834339.1	Uncultured fungus clone Z1E-9 18S ribosomal RNA	846	846	71%	0.0	97%
JF922020.1	Malbrancheacinnamomea strain CBS960.72 5.8S ribosomal RNA	616	616	49%	3.00E-172	99%
EF550978.1	Malbrancheaflava isolate MTCC 4887; 5.8S ribosomal RNA gene	558	558	51%	5.00E-155	94%
KP828175.1	Uncultured fungus clone CII-K39 5.8S ribosomal RNA	466	466	57%	3.00E-172	88%
KP278210.1	Gymnoascus sp. NZ-2015b strain S-5c 18S ribosomal RNA	466	466	81%	3.00E-172	82%
KP278209.1	Gymnoascus sp. NZ-2015b strain S-4c 18S ribosomal RNA	466	466	81%	3.00E-172	82%



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## CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest

## AUTHOR'S CONTRIBUTION

SA and VR were involved in collection of the samples and molecular analysis of the fungal species. VKR and RM have done the bioinformatic analysis of the data obtained from sequencing. SG was involved in conceptualization of idea and wrote the manuscript.

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## DATA AVAILABILITY

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

## ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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