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Lignocellulosic Enzyme Production by *Termitomyces* spp from Termite Garden

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Abstract In nature, microorganisms including fungi are the most efficient cellulose and hemicelluloses degraders through production of lignocellulosic enzymes. *Termitomyces* spp., a wild edible mushroom is well known for production of lignocellulosic enzymes. Twenty isolates of *Termitomyces* spp. were isolated at 10^{-4} dilution with pour plate method from different locations of termite mound soil of Tamil Nadu. Based on their morphological and cultural characteristics, the isolates were identified as *Termitomyces*. Growth conditions and cultural characteristics of *Termitomyces* spp. isolates were studied. The results showed that the isolates grew well in Malt extract agar medium with pH 5.5 when incubated at 27° C for 7 days. The isolates were screened for the activity of enzymes *e.g.,* cellulase, amylase, xylanase, polyphenol oxidase and peroxidase activities at seven different incubation periods. Maximum activity of the enzymes was observed on 12th day after inoculation which declined thereafter.

Keywords Hemicellulose, Lignocelluloses, *Termitomyces* spp

1. Introduction

About 40-60 million years before the advent of human agriculture, three insect lineages like Termites, Ants and Beetles independently evolved the ability to use fungi for food. Today out of the total more than 2600 sp. of termites, 330 are known to form an obligate symbiosis with the specialized fungi *e.g., Termitomyces* spp. [1]. On the termite comb white spherical yeast like colony fungal structures are commonly observed. These structures of *Termitomyces* have been described by various authors under different names. Heim [2] proved these structures exclusively as sporodochia which are found on the combs of Macro terminae and represent the anamorph of the genus *Termitomyces*. In plant cell walls hemicelluloses is a prime biomass reservoir. Agriculture based waste plant products have huge hemicelluloses biomass. Due to this biomass, more efforts have been taken to establish the standard method to degrade this hemicelluloses biomass in last decades [3]. The synergistic activities of termites and different lignocellulose enzymes complex are responsible for degradation of plant cell walls [4]. Makonde *et al*. 2014[5] described More than 60 *Termitomyces* taxa but only 18 species, collected from West Africa, are reasonably well known, the rest are either synonyms or badly described and difficult to identify. Commonly associated with woody debris and forest litter, these fungi are able to depolymerize, degrade and fully mineralize all cell wall polymers, including cellulose, hemicelluloses, and the normally rather recalcitrant polymer lignin [6].In addition, termites and other animals have made use of these capacities by utilizing microbial symbionts in the digestion of lignocellulosic food [7]. Different author suggests that *Termitomyces* spp. produce cellulases and xylanases which degrade cellulose and hemicellulose respectively [8, 9]. In addition, *Termitomyces* fungi degrade plant lignins to assist cellulose degradation by termites [10, 11]. Enzymes like cellulase, amylase and xylanase from *Termitomyces clypeatus* have been screened and studied for their commercial utility [12]. It is also amenable to large scale cultivation in a bioreactor and gives high productivity of CDH *Termitomyces albuminosus*, is a fungus that has a symbiotic relationship with termites, the so-called fungus-growing termites. Fungus-growing termites construct a fungus comb from plant litter, and cultivate *Termitomyces albuminosus* on this comb as food [2]. The fruiting bodies from inside the tunnels and bore through the very hard layer of inert matter, forcing their way through it with a special umbo [13]. *Termitomyces albuminosus* partially degrades plant materials, probably including lignin, to help digestion of cellulosic resources by termites [14]. Hence, the present study has been conducted to screen the *Termitomyces* spp. for lignocellulosic enzyme production.

2. Materials and Methods

2.1. Sampling Site

A macrofungi *Termitomyces* spp. and soils from the termite ecosystem were collected from different parts of Tamil Nadu during the monsoon time. The collected samples were taken to the laboratory for isolation purpose under aseptic condition.

2.2. *Termitomyces* cultures

The standard culture used in this study was *Termitomyces albuminosus* (MTCC 1366) obtained from Institute of Microbial Technology, Chandigarh.

2.3. Isolation of *Termitomyces* from termite mound

Termitomyces were collected from the fresh termite garden, washed with 0.5% sodium hypo chlorite for 2 minutes and then washed with sterile distilled water for 5 minutes. Finally rinsed with 0.05% sterile saline. The surface sterilized samples were inoculated in to PDA plates and incubated at room temperature. The plates were observed every day for appearance of fungal colony.

2.4. Isolation of *Termitomyces* from fruiting body

The mushroom tissue was used for isolation of fungi, following the method illustrated by Chang [15]. The mushrooms collected from fungus growing termite mound was washed and disinfected with 75% alcohol to remove soil debris. The volva was removed and the mushrooms were cut opened by hand longitudinally in halves. Small pieces were aseptically removed from the centre and inoculated on to PDA in which ampicillin (50 ppm) was added as antibacterial agent. Plates were incubated at room temperature (25±3^{o} C) in incubated shaker SIF 6000 R Vol 230VAC 50H$_{Z}$.

2.5. Purification

The different isolates obtained were purified by single hyphal tip method described by Rangasamy *et al.* [16]. A tip of actively growing mycelium from the individual culture was transferred to solidified malt extract agar (MEA) medium, subsequently to MEA slant and maintained at 4^{o}C.

2.6. Factors influencing the growth of *Termitomyces*

2.6.1. Carbon source

The *Termitomyces* isolates were grown in malt extract agar media with different carbon sources like glucose, sucrose, starch and malt extract (at the rate of 30g/L). The plates were incubated for 8 days at room temperature and observed for growth after incubation.

2.6.2. pH

Termitomyces isolates were inoculated in Malt extract media having five different pH ranges *e.g.,* 4.5, 5.5, 6, 7 and 8 and incubated for 8 days at room temperature and observed for their growth.

2.6.3. Temperature

Termitomyces isolates were inoculated in Malt extract media and incubated for 8 days at six different temperatures *e.g.,* 0^{o}C, 15^{o}C, 27^{o}C, 37^{o}C, 42^{o}C and 50^{o}C.

2.7. Characterization of *Termitomyces*

Characterization of *Termitomyces* was done by observing the morphological characters of the mushroom, colony morphology was studied.

2.8. Macro Morphological Characterization

Termitomyces was identified by the macro morphological characters described by Tibuhwa *et al.* [17] and Srivastava *et al.* [18]. Important characters used for identification were cap size, gills, colour, stipe and pileus.

2.9. Colony Morphology

The different *Termitomyces* isolates were identified by microscopic characterization in compound microscope Magnus MLX under 40x magnifications. *Termitomyces* isolates were differentiated by their colony characters for example size, shape, colour and consistency [19]. For microscopic identification, the isolates were grown by cover slipculture methods [20] in which individual cultures were transferred to the base of cover slip buried in MEA medium. The cover slip was removed after 8 days of incubation and morphological characters were observed for their mycelial structure, and spore arrangements in microscope. The observed morphology of the isolates was compared with the standard *T. albuminosus* MTCC 1366 for the presumptive identification of the isolates.

2.10. Screening of *Termitomyces* spp. for Biomass Production

All the isolates were inoculated in MEA (100 ml) containing different carbon source (Carboxy methyl cellulose, starch and xylose) and incubated in room temperature for 12 days. The initial weight of the culture and final weight was recorded and biomass was calculated. The isolates producing more biomass was used for the enzymatic studies.

2.11. Enzymatic Assays

The isolates screened based on the biomass production were used for the enzyme activity studies. The cellulolytic activity (Cellulase assay) was determined by colorimetric method [21] using the DNS reagent. The cultures were grown in malt extract broth and the activities were

measured at 3 days interval till 21st day after inoculation (0, 3, 6, 9, 12, 15, 18 and 21 days). Two ml of culture was centrifuged at 13,000 rpm for 5 minutes. From the culture supernatant, 0.1 ml was incubated with 0.9 ml of a CMC solution (1% w/v) in 50 mM sodium phosphate buffer (pH 7.0) and 0.25 ml distilled water at 40°C for 30 minutes. After incubation, the enzyme activity was stopped by adding 1.5 ml DNS-reagent. Then the tubes were placed in boiling water bath at 50°C for 15 minutes, cooled down to room temperature. Glucose, used as the standard was prepared at different concentrations 20,40,60,80 and 100 ppm and the assay was carried out as mentioned above. The OD of the sample and standard was immediately measured at 575 nm.

The results were interpreted from the standard graph and expressed in terms of µmol of glucose released/min/mg protein. Chen *et al.* [22]. β-Glucosidase activity was estimated at 50°C by measuring the release of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) prepared in citrate buffer [23]. For xylanase the assay mixture contained 0.1 ml enzyme solution, 0.1 ml xylan (10 mg / ml on 0.1N acetate buffer, pH 5.0) and 0.2 ml of the buffer.

The mixture was incubated for 30 min at 40°C and addition of 0.4 ml of alkaline copper reagents stopped the reaction. The mixture was kept for 10 min in a boiling water bath at 40°C and cooled. Then 0.2 ml of arsenomolybdate reagent was added. After 15 minutes, the mixture was centrifuged. Then the supernatant was placed in spectrophotometer at 540nm. Xylose, used as the standard was prepared at different concentrations 20,40,60,80 and 100 ppm and the assay was carried out.

Amylase assay was carried out by the method of Nelson [23] as modified by Somogyi [24] and also described earlier by Ghosh *et al*. [25]. The isolates were grown in malt extract media and the assay was carried out by measuring the amount of reducing sugar liberated at three days interval till 21st day after inoculation (0, 3, 6, 9, 12, 15, 18 and 21 days) from the samples. The assay mixture contained 0·02 ml of culture filtrate or enzyme solution, 0·2 ml of starch (soluble) suspension or solution (10 mg/ml in 0·1 M acetate bluffer, pH 5·0) and 0·18 ml of the buffer. Incubation was carried out for 30 min at 40°C and stopped by adding 0·4 ml of alkaline copper reagent. Glucose, used as the standard was prepared at different concentrations 20,40,60,80 and 100 ppm and the assay was carried out.

Poly phenol oxidase activity was determined by the method described by Zuber and Manibhushanrao [26]. For all the assays the cultures were grown in malt extract broth and the activities were measured at 3 days interval till 21st day after inoculation (0, 3, 6, 9, 12, 15, 18 and 21 days) Crude cell extract obtained from the *Termitomyces* culture grown in malt extract broth was centrifuged at 15,000 rpm for 4°C for 15 min and was used as enzyme source. 2.5 ml of 0.1 M- phosphate buffer (pH 6.0) and 0.3 ml of catechol solution (0.01 M) was pipette out in a cuvette. Spectrophotometer was set at 495 nm at 15,000 rpm for 4°C for 15 min. Peroxidase assay was done by the method described by Putter [27]. The assay mixture consisted of 3 ml of pyrogallol (0.05M), 0.5 ml of the enzyme source and 0.5 ml of 1% H_2O_2. The absorbance was measured in a spectrophotometer at 420 nm for every 30s upto 3 minutes.

3. Results and Discussion

Isolation of Termitomyces

Four isolates of *Termitomyces* spp. were obtained from the soil samples collected from different locations of Tamil Nadu and sixteen isolates were isolated from the fruiting bodies of *Termitomyces* collected during the monsoon period. Totally twenty isolates of *Termitomyces* were purified and designated with isolate numbers (Table 1).

Table 1. *Termitomyces* isolates obtained from termite mound samples collected fromdifferent locations.

Place of collection	Isolates	Colony characters on PDA medium
Sathyamangalam 11o 30' 18.94" N 77o 14' 17.74" E (Forest ecosystem)	Tm_1	White powdery growth
	Tm_2	White circular powdery mycelia
	Tm_3	White circular mycelia surrounded by slimy layer
TNAU (Cultivable land) 11o 07' 3.36" N 76o 59' 39.91"E	Tm_4	White mycelial growth
	Tm_5	White powdery growth with brown shade
	Tm_6	White mycelial growth with shrinkage
TNAU (Cultivable land) 11o 07' 3.36" N 76o 59' 39.91"E	Tm_7	White powdery growth
	Tm_8	White powdery mycelia
	Tm_9	Brown powdery mycelia
Thalavadi 9o 20' 59.99" N (Forest ecosystem) 76o 31' 59.99"E	Tm_{10}	Brown mycelia surrounded by white powdery growth
	Tm_{11}	White cottony mycelia
	Tm_{12}	White mycelial growth
	Tm_{13}	Brown powdery mycelia
	Tm_{14}	White powdery growth with brown shade
	Tm_{15}	White mycelial growth with shrinkage
	Tm_{16}	White powdery circular colony
Kovai courtallam 10o 56' 20"N (Forest ecosystem) 76o 41' 20"E	T_{17}	White circular powdery growth later turns brown
Pudukottai 10o 37'59.99"N (Waste land) 18o 07' 60.00"E	T_{18}	White circular mycelia later forms spore like structure
Aliyar 10o 28' 26.04"N (Cultivable land) 76o 58' 22.08"E	T_{19}	White circular powdery growth
Tirunelveli 8o 43' 48.00"N (Cultivable land) 77o 42' 0.00"E	T_{20}	White circular mycelia

T1-T16 isolates from fruiting bodies of *Termitomyces* collected from termite mound;
T17-T20 isolates obtained from termite mound soil.

Factors Influencing the Growth of *Termitomyces* Carbon Source

All carbon source used in the study supported the growth of *Termitomyces* spp in Czapek- dox medium whereas starch and malt extract favored the growth of *Termitomyces* spp compared to other carbon sources. However, malt extract appears to be the preferred carbon source for growth of *Termitomyces* spp. Glucose and sucrose supported the growth but the incubation period was more (i.e. 12 days) compared to other carbon sources. Growth of the isolates in different carbon sources were recorded and presented (Fig. 1a).

Figure 1a. Effect of carbon source on growth of Temitomyces

pH

The media with pH 5.5 favored the growth of the isolates followed by pH 4.5. Least growth was observed in the plates with pH 8. This experiment showed that pH 5.5 favored the growth of *Termitomyces*(Fig. 1b).

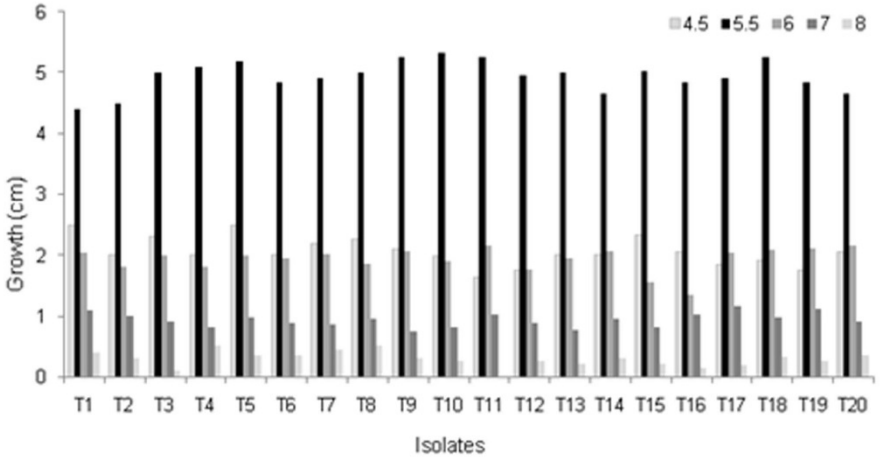

Figure.1b. Effect of pH on growth of Termitomyces

Temperature

The growth was better at 27ºC compared with other temperatures tested. This study showed that *Termitomyces* spp. preferred the optimum temperature of 27ºC for its growth (Fig. 1c).

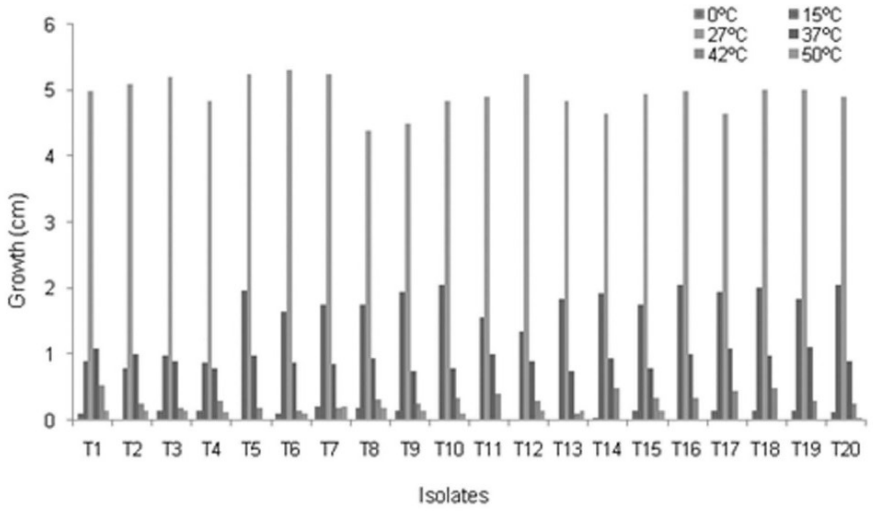

Figure.1c. Effect of temperature on growth of Temitomyces

Characterization of the Isolates

Macro Morphological Characterization

The colour of stalk was white having long stipe, colour of the cap was varying from dull white to brown.

Colony Morphology

The newest mycelia on the periphery of the culture were usually light and white in colour, while the more aged mycelium toward the centre of the culture were even darker and more differentiated. Mycelia aggregated into elevated forms forming cottony ball-like structures. These balls like structures become dark-brown on the surface of the stroma with age.

Micro Morphological Characterization

Under microscopic observation, *Termitomyces* spp. produced white, turning creamy, soft, hymenophoraltrama regular with thin walled parallel hyphae. Pileal surface had hyphae forming a hypodermal layer below the epicutis consisting of radially parallel, repent, narrow hyphae.

Screening of *Termitomyces* Spp. Based on Biomass Production

The experiments resulted in the maximum biomass production of 3.01g after 12 days of incubation in the liquid media with 1% CMC as carbon source. Only 7 isolates yielded more biomass among the twenty in all the three different carbon sources. Thus the seven isolates screened were used for enzyme activity studies (Fig. 2).

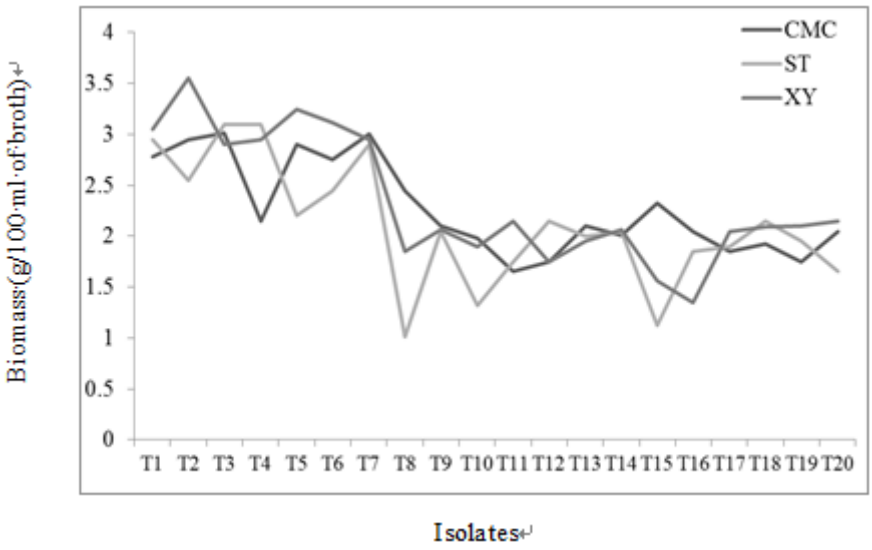

Figure 2. Biomass production of *Termitomyces* in different carbon sources. (CMC, Carboxy methyl cellulose; ST, Starch; XY, Xylose)

Studies on Enzymes Activity in *Termitomyces*

Cellulase Assay

The cellulase activity of *Termitomyces* isolates along with standard strain (*T. albuminosus* MTCC 1366) at different incubation periods was presented in Table 2**.** All the isolates tested and standard strain MTCC 1366 showed increased cellulase activity up to 12 days of incubation in Czapek-dox broth at room temperature under batch culturing condition and gradually decreased against the incubation time up to 21 days (Table 2). Among the isolates, Tm_3 recorded the maximum activity of 10.27 µmol of glucose released/min/mg protein at 12th day after inoculation followed by the standard strain (*T. albuminosus*MTCC 1366). Among the isolates, Tm_5 had least activity on 12th day (9.7 µmol of glucose released/min/mg protein).

Table 2. Cellulase activity of different *Termitomyces* isolates

	Cellulase activity (µ mol of glucose released/min/mg protein)							
Isolates	**0th day**	**3rd day**	**6th day**	**9th day**	**12th day**	**15th day**	**18th day**	**21st day**
T1	6.43 ±0.72c	6.93 ±0.54c	7.27 ±0.31c	7.90 ±0.41b	10.07 ±0.43a	8.17 ±0.72b	7.57 ±0.31c	6.10 ±0.66c
T2	5.50 ±0.4c	6.27 ±0.31c	6.97 ±0.12c	7.97 ±0.20b	9.87 ±0.26a	9.27 ±0.27a	7.87 ±0.29b	7.13 ±0.18b
T3	6.03 ±0.44c	6.50 ±0.29c	7.20 ±0c	7.90 ±0.35b	10.27 ±0.26a	8.87 ±0.40b	8.37 ±0.33b	6.80 ±0.86c
T4	5.83 ±0.83d	6.73 ±0.63d	7.60 ±0.35c	8.43 ±0.14b	9.93 ±0.34a	8.67 ±0.68b	7.43 ±0.31c	9.70 ±0.3a
T5	5.83 ±0.44d	6.53 ±0.26d	7.17 ±0.13c	8.13 ±0.19b	9.70 ±0.11a	8.93 ±0.15b	8.20 ±0.15b	7.50 ±0.15c
T6	6.43 ±0.55d	7.10 ±0.63c	7.47 ±0.29c	7.87 ±0.35c	10.20 ±0.4a	8.47 ±0.68b	7.50 ±0.57c	6.20 ±0.75d
T7	5.43 ±0.29c	6.50 ±0.25c	7.50 ±0b	8.63 ±0.13b	10.10 ±0.15a	9.00 ±0.21a	7.63 ±0.31b	6.90 ±0.2c
Ta	5.77 ±0.28d	6.47±0.21d	7.37±0.13c	8.27 ±0.15b	10.03 ±0.33a	9.30 ±0.33a	8.20 ±0.1b	7.57 ±0.28c

Ta –*Termitomyces albuminosus* (MTCC 1366). Values are mean ± SE

Means in a columan followed by a same letter(s) are not significantly ($p \leq 0.05$) different according to DMRT.

Xylanase Assay

All the seven isolates obtained from fruiting body and the standard strain (*T. albuminosus* MTCC 1366) exhibited xylanse activity (Table 3). The isolate Tm_4 showed maximum activity of 9.87µmol of xylose released/ min/ mg protein followed by the isolates Tm_6 and Tm_7 on 12th day of incubation, which was however on par with the standard culture (*T. albuminosus* MTCC 1366) recording the activity of 9.77 µmol of xylose liberated/min/mg protein on 12th day of incubation. In all the isolates, the maximum xylanase activity was recorded on 12th day and was decreased gradually over incubation.

Table 3. Xylanase activity of different *Termitomyces* isolates.

	Xylanase activity (µ mol of xylose released/min/mg protein)							
Isolates	**0th day**	**3rd day**	**6th day**	**9th day**	**12th day**	**15th day**	**18th day**	**21st day**
T1	6.87 ±0.46d	7.40 ±0.2c	7.77 ±0.23c	8.80 ±0.1b	9.67 ±0.18a	8.77 ±0.23b	8.23 ±0.14b	7.13 ±0.08c
T2	6.90 ±0.25c	7.37 ±0.2b	7.63 ±0.42b	8.43 ±0.56a	9.73 ±0.17a	8.97 ±0.03a	7.86 ±0.43b	6.47 ±0.51c
T3	6.93 ±0.13d	7.20 ±0.15c	7.90 ±0.15c	8.37 ±0.23b	9.60 ±0.1a	9.10 ±0.43a	7.60 ±0.35c	6.90 ±0.05d
T4	6.90 ±0.37c	7.53 ±0.24b	7.87 ±0.24b	8.60 ±0.20a	9.87 ±0.08a	8.90 ±0a	8.00 ±0.05b	7.06 ±0.08b
T5	6.97 ±0.17d	7.40 ±0.3c	7.77 ±0.46c	8.47 ±0.42b	9.70 ±0.11a	9.03 ±0.33a	7.36 ±0.31c	6.67 ±0.37d
T6	6.77 ±0.35c	7.37 ±0.27b	7.83 ±0.29	8.93 ±0.17a	9.77 ±0.16a	8.66 ±0.18a	7.80 ±0.15b	6.90 ±0.26c
T7	7.20 ±0.28b	7.50 ±0.26b	7.67 ±0.33b	8.23 ±0.12a	9.77 ±0.03a	8.60 ±0.15a	7.83 ±0.21b	6.93 ±0.03c
Ta	6.90 ±0.20c	7.67 ±0.24b	8.10 ±0.15b	8.73 ±0.23a	9.77 ±0.14a	8.97 ±0.26a	7.17 ±0.12b	6.80 ±0.23c

Ta – *Termitomyces albuminosus* (MTCC 1366). Values are mean ± SE.

Means in a columan followed by a same letter(s) are not significantly ($p \leq 0.05$) different according to DMRT

Amylase Assay

All the isolates exhibited higher activity on 12th day of incubation and results are presented in Table 3. Isolate Tm_6 showed maximum amylase activity of 14.5 µmol of glucose released/ min/ mg protein on 12th day of incubation, followed by T_3 (13.77 µmol of glucose released/ min/ mg protein). The standard culture showed least activity of 13 µmol of glucose released/ min/ mg protein on 12th day of incubation (Table 4).

Table 4. Amylase activity of different *Termitomyces* isolates.

	Amylase activity (µ mol of glucose released/min/mg protein)							
Isolates	**0th day**	**3rd day**	**6th day**	**9th day**	**12th day**	**15th day**	**18th day**	**21st day**
T1	8.93 ±1.0c	9.83 ±0.89c	10.83 ±1.4b	12.13 ±1.34a	13.73 ±1.62a	12.47 ±2.13a	10.37 ±1.25b	8.70 ±0.86c
T2	6.73 ±0.31e	7.97 ±0.43d	8.93 ±0.66c	9.87 ±0.73b	11.73 ±1.02a	9.77 ±1.47b	9.00 ±1.04b	8.27 ±0.99c
T3	9.67 ±0.84e	10.27 ±0.71d	11.20 ±0.98c	12.00 ±1.04b	13.77 ±0.72a	12.83 ±0.83b	10.70 ±0.15d	8.90 ±0.95e
T4	7.43 ±0.88e	8.57 ±0.99d	9.17 ±0.98c	10.67 ±1.46b	11.73 ±1.8a	10.00 2.29b	8.90 ±1.07d	7.80 ±0.90e
T5	8.50 ±1.01e	9.40 ±0.70d	10.17 ±0.66c	11.13 ±0.46b	13.10 ±0.25a	11.90 ±0.50b	10.33 ±0.46c	9.27 ±0.31d
T6	9.27 ±0.76e	10.37 ±0.72d	11.47 ±1.01d	12.60 ±1.0c	14.50 ±0.85a	13.50 ±0.8b	11.03 ±0.63d	8.70 ±0.62e
T7	6.8 ±0.40e	8.03 ±0.55d	8.93 ±0.71d	10.06 ±0.69b	11.33 ±1.23a	9.17 ±1.58c	8.33 ±1.15d	7.53 ±0.95e
Ta	8.87 ±0.77e	9.67 ±0.63d	9.67±0.71d	11.06 ±0.47b	13.00 ±0.2a	11.6 ±0.60b	10.23 ±0.37c	8.57 ±0.67d

Ta – *Termitomyces albuminosus* (MTCC 1366). Values are mean ± SE.

Means in a column followed by a same letter (s) are not significantly ($p \leq 0.05$) different according to DMRT.

Polyphenoloxidase Assay

The isolate Tm_7 exhibited the maximum activity of 3.80 OD change/min/mg protein on 12th day of incubation followed by Tm_2 (2.93 OD change/min/mg protein) (Table 5)**.** The standard culture exhibited mean enzyme activity of 2.91 OD change/min/mg protein. Least activity was observed in the isolate Tm_6 (2.73 OD change/min/mg protein) on 12th day of incubation.

Table. 5. Poly phenol oxidase activity of different *Termitomyces* isolates

	Poly phenol oxidase activity (OD change/min/mg protein)							
Isolates	**0th day**	**3rd day**	**6th day**	**9th day**	**12th day**	**15th day**	**18th day**	**21st day**
T1	1.06 ±0.08b	1.36 ±0.13b	2.20 ±0.55a	2.17 ±0.66a	2.87 ±0.89a	2.20 ±0.81a	1.17 ±0.20b	0.90 ±0b
T2	1.05 ±0.03b	1.87 ±0.08b	2.40 ±0.17a	2.60 ±0.45a	2.93 ±0.48a	2.67 ±0.29a	1.93 ±052b	1.20 ±0.4b
T3	1.57 ±0.32b	1.93 ±0.29b	2.57 ±0.34a	2.70 ±0.52a	2.90 ±0.72a	2.23 ±0.63a	1.06 ±0.12b	0.90 ±0.1c
T4	1.30 ±0.26c	1.6 ±0.25b	2.03 ±0.24a	2.08 ±0.54a	2.83 ±0.73a	1.80 ±0.55b	1.33 ±0.34c	0.80 ±0.05c
T5	1.07 ±0.1b	1.9 ±0.1b	2.37 ±0.23a	2.63 ±0.47a	2.87 ±0.51a	2.40 ±0.36a	1.43 ±0.53b	1.10 ±0.40b
T6	1.43 ±0.33b	1.87 ±0.27b	2.37 ±0.37a	2.00 ±0.58a	2.73 ±0.88a	1.83 ±0.69b	0.97 ±0.12c	0.83 ±0.03c
T7	1.50 ±0.25c	1.97 ±0.24c	2.43±0.29b	2.87 ±0.23b	3.80 ±0.15a	2.50 ±0.36b	1.70 ±0.35c	1.00 ±0.26d
Ta	1.80 ±0.11b	2.03 ±0.13b	2.43 ±0.12a	2.10 ±0.50a	2.91 ±0.13a	1.97 ±0.21b	1.20 ±0.15c	0.83 ±0.03c

Ta – *Termitomyces albuminosus* (MTCC 1366). Values are mean ± SE.

Means in a column followed by a same letter (s) are not significantly ($p \leq 0.05$) different according to DMRT.

Peroxidase Assay

The isolate Tm_4 exhibited more peroxidaseactivity (3.87 OD change/min/mg protein) followed by the isolate Tm_1 (3.70 OD change/min/mg protein) on 12th day of incubation (Table 6). The standard culture exhibited mean enzyme activity of 3.52 OD change/min/mg protein. Least activity was exhibited by the isolate Tm_5 (2.27 OD change/min/mg protein).

Table 6. Peroxidase activity of different *Termitomyces* isolates

	Peroxidase activity (OD change/min/mg protein)							
Isolates	0th day	3rd day	6th day	9th day	12th day	15th day	18th day	21st day
T1	1.37 ±0.13c	1.7 ±0.20c	1.77 ±0.12c	1.37 ±0.3c	3.70 ±0.35a	2.27 ±0.52b	1.50 ±0.25c	0.93 ±0.03d
T2	1.70 ±0.25b	1.87 ±0.27b	2.73 ±0.31a	2.40 ±0.50a	2.63 ±0.47a	1.73 ±0.14b	1.23 0.17b	0.93 ±0.08c
T3	1.83 ±0.23c	1.93 ±0.23c	2.43 ±0.17b	1.93 ±0.63	3.07 ±0.31a	2.33 ±0.69b	1.10 ±0.05c	0.83 ±0.03d
T4	1.47 ±0.24c	1.87 ±0.18c	2.13 ±0.33b	1.87 ±0.23c	3.87 ±0.06a	2.43 ±0.29b	1.70 ±0.15c	0.90 ±0d
T5	1.77±0.33b	2.2 ±0.20a	2.5 ±0.37a	2.43 ±0.53a	2.27 ±0.13a	1.67 ±0.14b	0.97 ±0.12c	0.83 ±0.12c
T6	1.67 ±0.08c	2.03 ±0.08b	2.23 ±0.31b	1.90 ±0.66c	3.60 ±0.20a	2.40 ±0.60b	1.33 ±0.24c	0.83±0.03d
T7	1.70 ±0.26c	1.96 ±0.13c	2.30 ±0.3b	2.10 ±0.2b	3.40 ±0.45a	2.00 ±0.2b	1.27 ±0.20c	0.90 ±0.05d
Ta	1.70 ±0.26c	2.13 ±0.26b	2.77 ±0.18b	2.77 ±0.43b	3.52 ±0.56a	2.13 ±0.53b	1.06 ±0.16c	0.87 ±0.06d

Ta – *Termitomyces albuminosus* (MTCC 1366). Values are mean ± SE.

Means in a column followed by a same letter (s) are not significantly ($p \leq 0.05$) different according to DMRT.

4. Discussion

The intriguing activities and properties of termites none seem as widely recognized or as often quoted as their ability to utilize wood as food source. Many species prefer wood that is partially degraded by the associative fungi [28]. Termites especially the sub family macroterminae is known to form symbiosis with *Termitomyces*. Members of the two major fungus-growing termite genera, *Macrotermes* and *Odontotermes*, process plant biomass in a similar way [29] involving two gut passages and external decomposition in fungal gardens. Old workers collect plant substrate and transport the young workers ingest the plant material along with asexual *Termitomyces* spores produced in fungal nodules in the mature parts of the fungal comb. This mixture passes through young termite gut (first gut passage), which possibly contributes to lignin cleavage [30]. Mushrooms have been reported to produce a wide range of secondary metabolites having high therapeutic values such as antioxidant, antitumor, antibacterial, antiviral, cholesterol lowering, hematological agents and immunomodulating properties [31]. Lignocellulose is a heteropolymer consisting mainly of three components, cellulose, hemicellulose and lignin [32,

33]. The characteristics of these components are summarized, with the major enzymes responsible for their degradation in the termite mound.

Isolation of *Termitomyces* spp from Termite Ecosystems

Termitomyces spp. has become highly valued, partially due to their rareness and difficulty in cultivation [34]. *Termitomyces* spp. remain elusive and even laboratory trials to culture and cultivate them are far from success stories. Pure cultures of *T. heimii* were obtained using tissue culture and spore inoculation methods. It was easily obtained when young sporophores are used [35]. In the present study, twenty isolates were obtained from the mushrooms and termite mound collected from different locations using tissue culture method generally enriched in Potato Dextrose Agar (PDA) medium. Since *Termitomyces* is a slow grower in PDA, a selective media namely malt extract medium was used which enhanced the growth of fungus. Malt extract media is recommended for the detection, isolation and enumeration particularly for fungi in various materials and cultivation of this mould for microbiological vitamins and other antimicrobial compounds study. PDA and Malt extract media supported the growth of *Termitomyces* mycelia than the other culture media [36]. *T. umkowaani* showed an increased growth rate of up to 18mm in Malt extract media in 5 days as compared to 12mm in [36].

Morphological and Cultural Characterization of the Isolates

A detailed classification of *Termitomyces* based on the macro and micro morphological characters was given by Tibuhwa *et al.* [17]. Macro morphologically, three groups were classified based on the cap size. Morphological characterization can be done based on shape of perforatorium, stipe length(cm), pileus length, margin of fruit body, colour of fruit body, gills, flesh, annulus, pseudorrhiza and spore print [18] .

In the present investigation, the mushrooms collected from different locations were studied for their cap size, colour of the fruiting body, gills, flesh, etc for the identification of the genus macro morphologically. Based on the observations, the colour of the cap was varying from white cap with brown centre to grey cap with brown centre. Other morphological characters were also studied and the genus of some of the collected mushrooms was found to be *Termitomyces.* In the present study, the microscopic observation of the isolates revealed that the isolates produced white, turning creamy, soft, hymenophoraltrama regular with thin walled narrow, parallel surface had hyphae forming a hypodermal layer below the epicutis consisting of radially parallel, repent, narrow hyphae.

Enzyme Production by *Termitomyces* spp.

Termitomyces clypeatus is known as a potential producer of different enzymes in culture media [37]. Production of lignocellulosic enzymes (cellulase, xylanase, amylase, poly phenol oxidase and peroxidase) by *Termitomyces* isolates in present study consistent with earlier studies. Different enzymes such as endo1,4-D-Xylanase, 1,4-D-Xylosidase, α-L-rabinofuranosidase, acetyl esterase, α-amylase, and amyloglucosidase were also purified from *T. clypeatus* [37]. Ghosh and Sengupta [12] screened the lignocellulolytic enzymes from *T. clypeatus* and found their commercial utility to produce cellulase, amylase and xylanase under submerged condition in good titre. Ghosh and Sengupta [38] further studied the production of extracellular xylanase and amylase under submerged condition of *T. clypeatus.*

The isolate T1 recorded maximum cellulose activity in present study accordance with the studies of Zhang *et al.* [39] who reported the production of lignocellulolytic enzymes *e.g.,* cellulase, xylanase, amylase, laccase and peroxidase production under submerged fermentation of *Termitomyces eurrhizus*. *Termitomyces* conidiophores associated with the fungus garden of *M. michaelseni* produces a cellulase and *Termitomyces* is a very good source for cellulose system [40]. Gupta *et al.,* 2014 reported that *Termitomyces* spp. OE147 has been identified as a fungus that fully mineralizes woody material and produces large quantities of CDH [16] unlike most of the cellulolytic microorganisms. However, *Termitomyces* spp. isolated from Termite *Ancistiermus cervithoras* and *Microterms toumodiens is* have very low enzymatic activities. This was supported by Sawhasan *et al.* where, *Termitomyces clypeatus* isolated from the comb of *Hypotermyces makhamensis* have very low cellulose activities. Production of lignocellulolytic enzymes are mostly influenced by various inorganic additives and temperatures [41, 42]**.** The mushroom fungus *Lentinus conatus* elaborated maximum production of endocellulase (12.75 U/ml) and exocellulase (8.30 U/ml) in liquid and solid substrates [43].

Xylanase catalyzes the hydrolysis of xylopyranosyl linkages of β 1, 4 xylan, a plant polysaccharide next to cellulose in abundance. Owing to the increasing biotechnological importance of thermostablexylanases, many thermophilic fungi have been examined for xylanase production [44]. All the *Termitomyces* isolates have xylanase activity in this study is in accordance with the finding of Mukherjee and Sengupta who reported that the mushroom *Termitomyces clypeatus* produced an endoxylanase (1,4- beta -D-xylanxylanohydrolase) in the presence of either dextrin or xylan as sole source of carbon. Lakshmanan *et al*. [45] studied the lignocellulolytic enzymes production by *Lentinus conatus* and *Pleurotus sajor-caju* with various inorganic additives and the results revealed maximum production by addition of inorganic additives. Gianni *et al.* [46] observed production of xylanolytic enzymes (304 U/g) by *Fusarium oxysporum* grown on corn stover in solid state fermentation. Yang *et al*. [47] also found the production of extracellular xylanase under optimized

condition yields 18580 U /g of xylan. Faulet *et al.* [48] indicated that thermostable xylanase from *Termitomyces* exhibited 80kDa by gel filtration at 65° C -70 °C and pH 5.0 - 6.0.

Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however enzymes from fungi have dominated applications in industrial sectors [49]. Kathiresan and Manivannan [50] reported α- amylase production by *Penicillium fellutanum* isolated from mangrove rhizosphere soil. Ghosh and Sengupta [36] purified amylase from the culture filtrate of *Termitomyces clypeatus* by ammonium sulphate precipitation DEAE-Sephadex chromatography and gel filtration. Specific activity of amylase was 3.38 (U/mg protein) in the culture filtrate.

Basidiomycetes, which cause white rot decay, are able to degrade lignin in lignocellulosic wastes. White rot fungi produce extracellular phenoloxidase, which are responsible for initiating the depolymerization of lignin [51]. Maximum laccase activity was exhibited by *P. djamor* in paddy straw substrate, 10 days after inoculation [52]. High enzymes activities of laccase, poly phenol oxidase and peroxidase were observed in *Lentinus conatus* at 25^{o} C, compared with *Pleurotus sajor-caju* [44]. Similar observation was also reported by Ganesh kumar [53] and Thanikaivelan [43].

Banik *et al.* [54] reported Cellobiase (b-glucosidase) is a well characterized enzyme of the cellulose multienzyme system which acts synergistically to degrade cellulose into constituent glucose subunits, the raw material thiol groups play a pertinent role in modulating the activity of both extracellular and intracellular cellobiase purified from the filamentous fungus *Termitomyces clypeatus* [54] and inclusion of reducing agents such as dithiothreitol (DTT), stimulated cellobiase activity in the fungus [55] Cellulase, lignin peroxidase and manganese peroxidase was assayed in *Aspergillus, Fusarium, Mucor, Rhizopus, Trichoderma* and some unidentified genera [56]. Lignin peroxidase activity was done using the method of Berridge [57]. Moreover, in present study all the *Termitomyces* isolates produced peroxidase. Glucose as carbon source and a mixture of yeast extract and peptone at the ratio of 3 to 5 as nitrogen source in a production medium were shown to produce highest peroxidase activity (73 U/ml) by *Arthromyces ramosus* [58].

5. Conclusions

Producing fuel ethanol from inedible and abundantly available cellulose biomass offers an important opportunity to sustainably produce alternative transportation fuel. Although significant progress has been made to reduce the manufacturing costs, widespread commercialization of this technology has not been realized. The biofuel industry needs way to combine integrated production technologies and produce value-added byprooducts. A milestone in the scientific development would be microorganisms that can convert lignocellulosic biomass into ethanol efficiently and therefore would enable consolidated bioprocessing to become commercially attractive. Moreover, the development of naturally lingo cellulolytic microorganisms could be resumed, because this might solve the problem as well or could at least improve our understanding cellulosomal enzymes and how they work together. Currently many efforts are devoted to developing new technologies in further decrease the cost of pretreatment and generate less toxic chemicals, higher sugar yield and higher value byproducts through microbial enzymes.

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