Eco. Env. & Cons. 28 (October Suppl. Issue) : 2022; pp. (S398-S404)
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ISSN 0971–765X

DOI No.: http://doi.org/10.53550/EEC.2022.v28i06s.067

Valorization of leaf litter by ligninolytic fungi from termite garden

V. Gomathi and J. Kavitha Mary

Department of Agricultural Microbiology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

(Received 4 March, 2022; Accepted 29 June, 2022)

ABSTRACT

The present study was focused on three indigenous litter dwelling ligninolytic fungi viz., *Termitomyces* sp., *Streptomyces* spp, and *Xylaria* sp. from the fungal comb of termite garden were selected to study their efficiency in decomposing leaf litter. Three different leaf littersuch as sapota, mango, and guava dried leaves from orchards were used as the solid substrates to determine the efficiency of selected potential ligninolytic fungal strains to decompose them.Under *in vitro* conditions, the culture grows at 30-32^{0}C, pH 7.1 after 48 h, a clear zone was observed by the Congo Red plate method. The compost pile was supplemented with an isolated microbial consortium with 50-60 % moisture levels for 90 days to get maturity. The activity of ligninolytic cellulose (110 -240 µg $g^{-1}day^{-1}$) was observed higher in *Termitomyces*, whereas amylase in *Streptomyces* spp (950- 1370 µg g^{-1} day^{-1}) and dehydrogenase in *Xylaria* (540-880 µg $g^{-1}day^{-1}$).

Key words : *Termitomyces, Litter degradation, Cellulase, Amylase, Dehydrogenase.*

Introduction

Fungi is considered the key player in litter decomposition due to its ability to produce a wide range of extracellular enzymes. Most efficient litter degraders are saprotrophic Ascomycetes and Basidiomycetes. Lignocellulose decomposers belonging to Basidiomycetes namely, *Trametes versicolor, Collybia dryophila, C. peronata, Cyathus striatus, Clito cybegibba, Micromphale sp*. and *Mycena polygramma* were found to be efficient decomposers of larch litter that attacked both lignin and carbohydrates (Osono *et al*., 2003).

Brown-rot fungi selectively degrade the cellulose and hemicellulose in the litter, leaving behind the more recalcitrant lignin. Brown-rot fungi of importance include Serpula lacrymans, Fibroporia vaillantii, Coniophora puteana, Fomitopsis pinicola, Schizophyllum commune, Fomes fomentarius, etc. (Hammel *et al*., 2002). Whereas white-rot fungi degrade cellulose, hemicellulose and lignin at approximately equal rates. The decayed wood is pale in color, light in weight, and has a stringy texture. White rot fungi are the only organisms that can completely degrade lignin. Examples: *Ganoderma lucidum, Phanerochaete chrysosporium, Armillariella mellea, Hypholoma fasciculare* and Flammulina velutipes (Boerjan *et al*., 2003). Soft-rot fungi secrete cellulase from their hyphae, an enzyme that breaks down cellulose in the wood. Examples of soft-rot-causing fungi are Chaetomium, Ceratocystis, *Kretzschmaria deusta, Lulworthia, Halosphaeria,* and *Pleosporain.*

Termitomyces a basidiomycetes fungus has a symbiotic relationship with *Macrotermitinae* and grows only on fungus combs in their nests. It exists on the comb as mycelium, a small, white, round asexual fruiting structure called myocytes (Heim, 1940). This

symbiotic fungus played a different role in termite nutrition by providing cellulase and xylanase (Martin and Martin, 1978) by degradation of cellulosic wastes through extracellular enzymes.

Various research studies support the high cellulose degradation potential of microbes from Actinomycetales. *Streptomyces* is the most abundant hemicellulases producer among actinomycetes. Boroujeni *et al.* (2012) reported that all of the isolated hemicellulolytic actinomycetes were found to belong to the *Streptomyces* genus. Xylanase has been successfully purified from Streptomyces sp. E-86 and characterized for its xylanolytic activity (Kusakabie *et al.*, 1977). Xylanase has also been produced from other strains of *Streptomyces* sp. such as *Streptomyces* sp. strain C1-3 (Maryandani, 2007) and *Streptomyces* sp. CD3 (Sharma and Bajaj, 2005).

Ascomycetes of the family Xylariaceae have been reported to cause a special type of extensive wood-rot referred to as soft-rot type II (Blanchette, 1995). These fungi can degrade both polysaccharides and lignin, although, in contrast to white-rot basidiomycetes, the middle lamella is not attacked (Sutherland and Crawford, 1981). Despite their extensive occurrence in forests, there are only a few reports on the ligninolytic potential of Xylariaceae

Composting has gained renewed attention as an alternative technique for solid organic waste management. Microbes play an active role in composting process, because of their unique property of degrading enzymes. The microorganisms isolated from the termite mound ecosystem produce many extracellular enzymes and play a main role in lignin degradation. This present research focused on using microbes that are isolated from the termite mound ecosystem for leaf litter degradation.

Materials and Methods

The Fungal comb of Macrotermitinae was collected from the termite mound at five different locations inthe Western Ghats of Tamil Nadu.The samples were taken to the laboratory under aseptic conditions. The collected samples were analyzed for their physical, chemical, and biological properties.

Isolation of microorganism from fungal comb:

Fungi and actinomycetes were isolated from the fungal comb by dilution plate's method in respective media. The *Streptomyces* spp., *Termitomyces* sp., and *Xylaria* sp.were isolated from the termite mound, by pour plate technique using nutrient agar media. All the plates were incubated at 28-30^{0}C for 3-5 days, and counts were made. The results were expressed as several organisms per gram of sample on a dry weight basis.

Isolation of *Termitomyces*

Fungus combs were collected from different parts of the Western Ghats of Tamil Nadu. The fungus combs were taken to the laboratory with care for isolation by using specially modified media (General Soil fungus Media). *Termitomyces* were isolated and enriched in different substrates *viz*., maize cob, paddy straw, and coir. The culture preferred coir (5%) as substrate, temperature 30-32 ± 1 °C, pH 4-4.5, and relative humidity 85-90% for its effective growth under microclimatic conditions.

Chemical compositions of the fungus comb

The chemical composition like moisture content, pH,carbohydrate and lignin ratio, and major nutrients like nitrogen,phosphorus, and potassium were estimated in the collected fungal comb as per the standard procedure.

Determination of carbohydrate content in fungus comb:

The total carbohydrate content of the fungal combs was estimated by the Anthrone method. The samples were hydrolyzed with HCl and neutralized with sodium carbonate. The volume was made up to 100 ml and centrifuged, 0.5 and 1.0 ml of aliquots were taken from the supernatant for analysis. The number of carbohydrates present in the sample was calculated using glucose as standard.

Determination of lignin content in fungal comb

The lignin content in the fungal comb was determined by the gravimetric method. One g of fungal comb, 5 ml of conc.H_2SO_4, and 50 ml of 37% HCl were taken and kept for 16 h at 25 ^{0}C. The mixture was transferred into a 1-liter flask with 450 ml distilled water and boiled for 10 min and filtered through a Geena G3 glass filter. The acidic residues were washed to neutrality with distilled water, then dried at 105 ^{0}C and weighed. The results were expressed as carbohydrate /lignin ratio.

Estimation of major nutrients in fungal comb

The fungal comb was dried, powdered and 0.5 g of representative sample was taken for the estimation

of major nutrients. The samples were digested with diacid for the estimation of total nitrogen and with triple acid for the estimation of phosphorus and potassium. The total nitrogen content (Humphries, 1956), phosphorus content (Jackson, 1973), and potassium content (Stanford and Engfish, 1949) were estimated as per the standard procedure.

In vitro degradation efficiency of amylose and cellulose

The well was formed on a Petri dish containing CMC agar media by using a cork borer. To that the microbial cultures 0.1 ml was transferred into the well. The plates were incubated at room temperature for 48 h for *Bacillus* spp. and *Streptomyces* spp. and one week for *Termitomyces* fungi, and then flooded with 1% Congo red solution for 15 min followed by destaining with 1 M $NaCl_2$ solution. The zone formation around the well was examined. For determining the amylase activity microbial culture was plated on a petri dish containing 2 % starch agar media, incubated at room temperature. Then it was flooded with iodine solution. The clear zone formation around the colony was examined. From that the efficient isolates which degrade the cellulose and amylose were selected and used for leaf litter degradation.

Leaf litter degradation

For leaf litter degradation, the dried leaves were collected from the orchard. Composting was done under the pit method. For the pit method, one square meter pit was formed to fill the sand to the depth of 5 ". Two kg of the substrate (dried leaves) was spread uniformly in the pit and 4 g of macerated fungal consortium and 20 g of $CaCO_3$ was repeated in alternating layer stacked up to the height of 1 m and the pit was allowed to decompose for 90 days till it gets complete maturity.

Composting parameters

The samples were collected on the 30^{th}, $60^{th,}$ and 90^{th} days and the composting parameters like moisture content, pH, electrical conductivity, and enzymatic activity like amylase, cellulose, and dehydrogenase were estimated.

Enzymatic activity

Assay of Cellulase activity

One g of compost sample was taken in Erlenmeyer flask to that 1 ml of phosphate buffer (pH 6.0) and 2 drops of toluene were added. Then 1 ml of one percent carboxymethylcellulose, 3 ml of distilled water were added. The flask was stoppered and incubated at room temperature for 24 h. The supernatant solution was taken and the samples were measured for reducing sugar activity. The results were expressed as µg of glucose released per g of samples for 24 h incubation.

Assay of amylase

Amylase activity was assayed by extracting 1g of sample material with 5-10 volumes of ice-cold 10mM calcium chloride solution overnight at 4 °C and centrifuged at 54,000 g at 4 °C for 20 min. The supernatant is used as an enzyme source. 1 ml of starch solution and 1 ml of the properly diluted enzyme was pipette out in a test tube and incubated at 27 °C for 15 min. Then the reaction was stopped by the addition of 2 ml of dinitro salicylic acid reagent and heated the solution in a boiling water bath for 5 min, while the tubes were warm, 1 ml of potassium sodium tartrate solution was added. The contents of the tube were cooled in running tap water. The volume was made up to 10 ml with water. The absorbance was recorded at 560 nm. Amylase activity is expressed as µg of maltose produced during 24 h of incubation with 1% starch.

Assay of dehydrogenase

The dehydrogenase activity of the compost sample was measured by the spectrophotometric method. Compost samples were extracted with 0.2 ml of Triphenyltetrazolium chloride and 0.5 ml of 1glucosecose in screw-capped test tubes and then incubated at 37 °C for 24 h. after the incubation 10 ml of methanol was added and then allowed for 6 h. the intensity of red color was measured at 450 nm. The amount of Triphenylformazan (TPF) produced was calculated with reference to the calibration curve prepared from the TPF standard.

Results and Discussion

Microbial population in fungus combs

Fungus combs of different locations were collected and analyzed for their microbial population. The result showed that the bacterial population of the fungus combs ranged from 25.6-36.2 x 10^6 CFU / g sample dry weight. The fungi and actinomycetes populations ranged from 92.8-112.8 x 10^4 and 8.4 – 13.1 x 10^3CFU / g sample dry weight, respectively.

The results indicated that the fungal population was higher when compared to bacteria and actinomycetes (Table 1). This showed that an acidic environment in the fungus comb reduces the population of bacteria. The isolated bacterial population was identified as *Bacillus, Azotobacter*, *Beijerinckia,* and *Pseudomonas*. Fungi were identified based on morphological characteristics as *Dermitomyces*, *Aspergillus, Penicillium, Trichoderma*, *Fusarium, Xylaria,* and

Table 1. Analysis of the microbial population in fungus combs

Location	CFU/g sample on a dry weight basis		
	Bacteria x 10^6	Fungi × 10^3	Actinomycetes × 10^3
1	36.2	102.6	8.4
2	32.4	92.8	13.1
3	25.6	112.8	11.5
4	42.5	96.5	10.2
5	82.8	108.7	14.4
6	79.4	84.6	11.5

Table 2. Estimation of moisture content and pH of the fungus comb

Locations	Fresh comb	
	Moisture content	pH
1.	51.23	4.6
2.	48.50	4.4
3.	49.85	4.5
4.	46.92	4.4
5.	52.80	4.8
6.	50.64	4.6

the actinomycetes as Streptomyces.

Chemical composition of the fungus combs

Moisture percentage and pH in the collected samples were estimated as per the standard procedure. The results revealed that the moisture content of the fungus combs ranged from 46.92-52.80 % and the pH varied from 4.4-4.8. Though there was not much difference between samples, the highest moisture content and pH were recorded in the sample from location 5 (Table 2). The low pH in the fungus combs wood tends to prevent the development of bacteria, which favors the fungal population (Table 2).

Carbohydrate and lignin content of fungus comb

The collected fresh comb and old comb were assayed for carbohydrate (C) and lignin (L) ratio. The results indicated that C: L ratio was higher in the old comb when compared to the new comb. The C:L ratio of the old comb varied between 1.55-2.08 (w/w)

Table 3. Analysis of carbohydrate and lignin content of fungus comb

Locations	C/L ratio (w/w)	
	New comb	Old comb
1	0.78	1.98
2	0.64	1.67
3	0.60	1.55
4	0.86	2.08
5	0.84	2.02
6	0.76	1.90

and the C:L ratio of the New comb varied between 0.6-0.86 (w/w) (Table 3).

Nitrogen, phosphorus, and potassium content in fungus comb

The major nutrients like nitrogen, phosphorus, and potassium content of fungus comb were estimated. The results showed that the nitrogen content of fungus combs varied from 0.75-1.8% on a dry weight basis. The phosphorus and potassium contents of fungus combs varied from 0.175-0.193% and 0.075 to 0.08% on a dry weight basis, respectively (Table 4).

Many different species of fungi were also isolated from the fungus comb reported by Batra and Batra, (1966). In the active nests of mycelium, the myocytestes of Termitomyces are the only visible fungal growth, whereas when the comb is removed from the nest, it is soon enveloped by the growth of other fungi reported by Petch (1906). Neither the food store nor the nest structure, consisting of soil moistened with saliva, support more fungal growth. It is suggested that the development of other fungi

Table 4. Analysis of nitrogen, phosphorus, and potassium content in fungus comb

Locations	Nutrient (%) on a dry weight basis		
	N	P	K
1.	1.76	0.172	0.076
2.	1.68	0.175	0.075
3.	0.75	0.168	0.077
4.	1.78	0.193	0.069
5.	1.06	0.186	0.078
6.	1.80	0.177	0.080

on the comb is prevented by the mechanical activity of the termites, inhibition by the termite secretion (Batra and Batra, 1966), nest microclimate (Sands, 1969), antibiotic production, and the chemical composition of the comb (Grasse, 1944).

Thomas (1987) reported that pH of the fungus comb (4.1-4.6) and food store (5.3-5.8) were suitable for the growth of the fungi, with the lower pH of the fungus comb also being suitable for the activity of Termitomyces cellulases. The moisture content of the fungus comb (36-58%) was suitable for wood-rotting fungi. This was also suggested by Grasse (1944). The pH ranged from 3.9 to 4.35 suitable for Termitomyces cellulases for its activity (Martin and Martin, 1978). The results of the study on carbohydrate and lignin content estimation were agreed with studies conducted by Hyodo *et al.* (2003) that in *Macrotermes* spp., the carbohydrate lignin ratio of the combs increased with increased comb age, but it decreased or remained the same in *Odontotermes spp., H. makhamensis, A. pakistanicus,*and *P. militaris*. This efficient degradation of lignin in fungus combs has also been reported for *M.gilvus* and *M.carbonarius* (Hyodo *et al*., 2000). In terms of delignification, the high carbohydrate content in the primary feces may be very important, since white-rot fungi which include *Termitomyces* spp. are not able to use lignin alone as growth substrate, so other carbon and energy sources, such as glucose and cellulose are required (Kirk *et al*., 1978). From the ecosystem point of view, we can note that by associating with lignin decomposer, the fungus-growing termites make it possible to utilize lignocelluloses nearly completely, reflected in the small volume of their final faces (Darlington, 1994) and therefore, play a dominant role in decomposition processes in many areas of the tropics (Abe, 1980; Buxton, 1981).

Fungi serve as nitrogen-rich food, which is advantageous because the dead plant consumed by termites contains very little nitrogen (Matsumoto, 1976; Collins, 1983). The results revealed that the nitrogen content was high when compared to phosphorus and potassium in the fungus combs. The occurrence of lignin and polysaccharide degradation in the fungus comb during aging is predicted to be accompanied by nitrogen enrichment by eliminating carbon from the substrate. However, there is no evidence to support this prediction. The published results from *M.subhyalinus* (Abo-khatwa, 1977), *M. natalensis,* and *M. ukuzii* (Rohrmann, 1978) revealed that the nitrogen concentration in the old comb is lower than that in the fresh comb or unchanged. Nitrogen depletion during aging might be caused by the uptake of nitrogen by conidia in the mature comb (Rohrmann, 1978). Thus, it is unlikely that the fungus-growing termites selectively consume the old comb based on their nitrogen requirement alone, although the nitrogen concentration of the whole fungus comb is high, relative to the plant litter collected by the termites and the fungus comb produces conidia rich in nitrogen which is consumed to support larvae and nymphs (Collins, 1983). Therefore, the fungus comb is suggested to serve as the most suitable food, because it combines general nitrogen enrichment with specific accessibility of cellulose for digestion.

Composting is a biological process, it involves many microorganisms. The evaluation of compost has focused on compost maturity for the completion of the composting process. The important criteria used for determining composting maturity are moisture, pH, electrical conductivity, temperature, and enzymatic activity. The pH, temperature, moisture content of the compost sample was presented in Table 5. Compost microbes require a moist environment as they live in the water films surrounding composting organic matter particles. The results revealed the moisture content of the leaf litter compost ranged from 45 to 60 % for TLC (litter compost inoculated with *Termitomyces*, *Streptomyces* spp., *Bacillus* spp.) and 49 to 65% for LC (uninoculated). The optimum pH for TLC was about 5.93-6.2 and 6.1-6.9 for LC.

The enzymatic activity of the microbes played an important role in the formation of compost. The important implication of microbes in the composting is a fragmentation of high molecular weight components, which cannot pass through the cell wall into simpler ones by secreting the extracellular enzymes. In the initial stages of composting, bacteria utilize

Table 5. Composting parameters of the leaf litter compost

Sl. No.	Treatments	Moisture content (%)	pH	Ec (ds cm^{-2})
1	TLC 30	60	5.93	0.9
2	TLC 60	54	6.2	1.1
3	TLC 90	45	6.7	1.2
4	LC 30	65	6.9	0.7
5	LC 60	51	6.5	0.78
6	LC 90	49	6.1	0.92

LC- Litter compost, TLC- Litter compost + *Termitomyces*

the simple, easily degradable organic substances (Strom, 1985); it may also attack more complex material or may use substances released from the less degradable substances due to extracellular enzyme activities of other organisms. The remaining substances like cellulose or lignin, are degraded by fungi and actinomycetes (De Bertoldi *et al*., 1983).

In this study, the production of extracellular enzymatic activity of the *Termitomyces* fungi and the *Streptomyces* spp. was tested by *in vitro* method for cellulase and amylase degradation. The evolution of the particular enzymatic activity was tested by providing specific substrates like carboxymethyl cellulose for cellulose degradation and starch for amylose degradation. In our experiment in the Congo red method 3 mm diameter of the clear zone around the growth of the *Streptomyces* after 48 h, indicating the degradation of cellulose. In starch agar media also 4.5 mm diameter clear zone was obtained in the *Termitomyces* fungi after one week, due to the slow growth of the *Termitomyces* fungi.

During the initial stages of leaf litter decomposition microbial colonization alters the chemical and physical nature of the leaf resources, leaves become softened, rise in nitrogen, ATP content, respiratory rate, and the activity of degradative enzymes associated with leaf matrix (Keller *et al*., 1983). In composting, the soluble organic matter in the starting material is initially assimilated by the microorganisms. Once the soluble organic matter is used up, microorganisms produce a hydrolytic enzyme to depolymerize the larger compounds, i.e. lignin, cellulose, hemicellulose to smaller compounds that are water-soluble, then these water-soluble compounds dissolve in water, and are finally assimilated by the microorganisms (Tiquia *et al*. 2002). The *Termitomyces* fungi, *Streptomyces* spp. and *Xylaria* spp. used up a large quantity of soluble organic matter from the leaf litter therefore the compost inoculated with these microorganisms showed very high extracellular activity during composting when compared to that of control.

Table 2. Enzyme activity of the leaf litter compost

Treatments	Cellulase ($\mu g\ g^{-1}day^{-1}$)	Amylase ($\mu g\ g^{-1}day^{-1}$)	Dehydrogenase ($\mu g\ g^{-1}day^{-1}$)
TLC 30	110	950	540
TLC60	240	1370	880
TLC 90	210	1180	620
LC 30	70	520	380
LC 60	100	736	600
LC 90	108	603	523
Leaf	90	310	200

LC- Litter compost, TLC- Litter compost + *Termitomyces*

The present study revealed that the cellulase and amylase activity was more in the compost incorporated with *Termitomyces* fungi, *Xylaria* spp., and *Streptomyces* spp., which were isolated from the termite mound ecosystem. When compared to that control amylase showed maximum activity than cellulase.

For detecting composting maturity, stability of dehydrogenase activity has been used as an indicator, it is also used as a measure of detecting total heterotrophic microbial population. (Tiquia *et al*., 2002). It is also used as a total biological activity of the soil. In this study, the dehydrogenase activity of the compost inoculated with *Termitomyces* fungi, *Streptomyce*s, and *Xylaria* showed higher activity (540 to 880 ml^{-1} /day) compare to that of control(380 to 600 $\mu g\ ml^{-1}$/day). The results showed the enzyme activity initially increased, then during the 60^{th} day it showed maximum activity then it was decreased which indicated the compost maturity (Table 6).

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